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(54) Oligonucleotides and method for detecting bacteria

(57) A synthetic oligonucleotide which is complementary to a nucleotide sequence of a gene selected from the group consisting of the *Shiga* toxin gene of *Shigella* species, the *ipa*H gene of *Shigella* species and EIEC, the *inv*E gene of *Shigella* species and EIEC, the *ara*C gene of *Salmonella* species, the Verocytotoxin-1 gene of EHEC or VTEC, the Verocytotoxin-2 gene of EHEC or VTEC, the toxic shock syndrome toxin-1 gene of *Staphylococcus aureus*, the *ctx* gene of *Vibrio cholerae*, and the enterotoxin gene of *Clostridium perfringens*; a method for detecting a bacterial strain by amplifying a region of the above gene by PCR using the above oligonucleotides as primers and detecting the amplified region; and a kit for the detection of the bacterial strain.

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 11 6416

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X A	EP-A-0 409 159 (SHIMADZU CORPORATION) 23 January 1991 * the whole document *	3,13-17 27-31, 37-41	C12Q1/68
X A	DATABASE WPI Week 9202 Derwent Publications Ltd., London, GB; AN 92-012715 & JP-A-03 262 500 (NIPPON SHOJI KK) , 22 November 1991 * abstract *	1-4,11, 12 9,10	
X	JOURNAL OF CLINICAL MICROBIOLOGY, vol. 29, no. 9, September 1991 WASHINGTON US, pages 1910-1914, M.P. JACKSON 'Detection of Shiga toxin-producing Shigella dysenteriae type 1 and Escherichia coli by using polymerase chain reaction with incorporation of digoxigenin-11-dUTP'	1-4	
A	* the whole document *	5,6	C12Q
X	THE JOURNAL OF INFECTIOUS DISEASES, vol. 167, no. 2, February 1993 CHICAGO US, pages 458-461, O. SETHABUTR ET AL. 'Detection of Shigellae and enteroinvasive Escherichia coli by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery' * the whole document *	1-4,7,8	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	18 March 1996	De Kok, A

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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D : document cited in the application
L : document cited for other reasons

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
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A	* the whole document *	5,6, 18-26	
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 56, no. 6, June 1990 WASHINGTON US, pages 1536-1540, K.A. LAMPEL ET AL. 'Polymerase chain reaction for detection of invasive Shigella flexneri in food'	1-4	
A	* the whole document *	7-10	
X	THE JOURNAL OF INFECTIOUS DISEASES, vol. 162, no. 5, November 1990 CHICAGO US, pages 1195-1198, D.R POLLARD ET AL. 'Differentiation of Shiga toxin and Vero cytotoxin type 1 genes by polymerase chain reaction'	1-4	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	* the whole document *	18,19	
A	EP-A-0 355 989 (SHIMADZU CORPORATION) 28 February 1990 * the whole document *	13-17	
A	DATABASE WPI Week 9249 Derwent Publications Ltd., London, GB; AN 92-401805 & JP-A-04 297 488 (SHIONOGI & CO LTD) , 21 October 1992 * abstract *	18-26	

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A	DATABASE WPI Week 9249 Derwent Publications Ltd., London, GB; AN 92-401806 & JP-A-04 297 489 (SHIONOGI & CO LTD) , 21 October 1992 * abstract * ---	18-26	
A	EP-A-0 526 876 (WAKUNAGA SEIYAKU KABUSHIKI KAISHA) 10 February 1993 * the whole document * ---	27-31	
A	DATABASE WPI Week 9347 Derwent Publications Ltd., London, GB; AN 93-373605 & JP-A-05 276 996 (TOYOBO KK) , 26 October 1993 * abstract * ---	32-36	
A	PATENT ABSTRACTS OF JAPAN vol. 16 no. 338 (C-0965) ,22 July 1992 & JP-A-04 099488 (UNITIKA LTD) 31 March 1992, * abstract * ---	32-36	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	PATENT ABSTRACTS OF JAPAN vol. 15 no. 193 (C-0832) ,17 May 1991 & JP-A-03 049699 (SHIMADZU CORP) 4 March 1991, * abstract * --- -/--	37-41	

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
P,X	DATABASE WPI Week 9428 Derwent Publications Ltd., London, GB; AN 94-230239 & JP-A-06 165 698 (SHIMADZU CORP) , 14 June 1994 * abstract * ---	32-36	
L	DATABASE WPI Week 9514 Derwent Publications Ltd., London, GB; AN 95-100814 & JP-A-07 008 279 (SHIMADZU CORP) , 13 January 1995 * abstract * ---	32-36	
L	DATABASE WPI Week 9514 Derwent Publications Ltd., London, GB; AN 95-100815 & JP-A-07 008 280 (SHIMADZU CORPORATION) , 13 January 1995 * abstract * ---	1-6, 18-26	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
L	DATABASE WPI Week 9503 Derwent Publications Ltd., London, GB; AN 95-018273 & JP-A-06 303 976 (SHIMADZU CORP) , 1 November 1994 * abstract * -----	27-31	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	18 March 1996	De Kok, A

CATEGORY OF CITED DOCUMENTS

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European Patent Office

EP 94116416

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid,

namely claims:

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

☒ LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions,

namely:

1. Claims 1-4 partly, 5-12: Method and oligonucleotides for the detection of Shigella species and enteroinvasive Escherichia coli.
2. Claims 1-4 partly, 13-17: Method and oligonucleotides for the detection of Salmonella species.
3. Claims 1-4 partly, 18-26: Method and oligonucleotides for the detection of enterohemorrhagic or verocytotoxin producing Escherichia coli.
4. Claims 1-4 partly, 27-31: Method and oligonucleotides for the detection of Staphylococcus aureus.
5. Claims 1-4 partly, 32-36: Method and oligonucleotides for the detection of Vibrio cholerae.
6. Claims 1-4 partly, 37-41: Method and oligonucleotides for the detection of Clostridium perfringens.

☒ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respects of which search fees have been paid,

namely claims:

☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims,

namely claims

GENOME METHODS

Real Time Quantitative PCR

Christian A. Heid,[1] Junko Stevens,[2] Kenneth J. Livak,[2] and P. Mickey Williams[1,3]

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We have developed a novel "real time" quantitative PCR method. The method measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan Probe). This method provides very accurate and reproducible quantitation of gene copies. Unlike other quantitative PCR methods, real-time PCR does not require post-PCR sample handling, preventing potential PCR product carry-over contamination and resulting in much faster and higher throughput assays. The real-time PCR method has a very large dynamic range of starting target molecule determination (at least five orders of magnitude). Real-time quantitative PCR is extremely accurate and less labor-intensive than current quantitative PCR methods.

Quantitative nucleic acid sequence analysis has had an important role in many fields of biological research. Measurement of gene expression (RNA) has been used extensively in monitoring biological responses to various stimuli (Tan et al. 1994; Huang et al. 1995a,b; Prud'homme et al. 1995). Quantitative gene analysis (DNA) has been used to determine the genome quantity of a particular gene, as in the case of the human *HER2* gene, which is amplified in ~30% of breast tumors (Slamon et al. 1987). Gene and genome quantitation (DNA and RNA) also have been used for analysis of human immunodeficiency virus (HIV) burden demonstrating changes in the levels of virus throughout the different phases of the disease (Connor et al. 1993; Piatak et al. 1993b; Furtado et al. 1995).

Many methods have been described for the quantitative analysis of nucleic acid sequences (both for RNA and DNA; Southern 1975; Sharp et al. 1980; Thomas 1980). Recently, PCR has proven to be a powerful tool for quantitative nucleic acid analysis. PCR and reverse transcriptase (RT)–PCR have permitted the analysis of minimal starting quantities of nucleic acid (as little as one cell equivalent). This has made possible many experiments that could not have been performed with traditional methods. Although PCR has provided a powerful tool, it is imperative that it be used properly for quantitation (Raeymaekers 1995). Many early reports of quantitative PCR and RT–PCR described quantitation of the PCR product but did not measure the initial target sequence quantity. It is essential to design proper controls for the quantitation of the initial target sequences (Ferre 1992; Clementi et al. 1993).

Researchers have developed several methods of quantitative PCR and RT–PCR. One approach measures PCR product quantity in the log phase of the reaction before the plateau (Kellogg et al. 1990; Pang et al. 1990). This method requires that each sample has equal input amounts of nucleic acid and that each sample under analysis amplifies with identical efficiency up to the point of quantitative analysis. A gene sequence (contained in all samples at relatively constant quantities, such as β-actin) can be used for sample amplification efficiency normalization. Using conventional methods of PCR detection and quantitation (gel electrophoresis or plate capture hybridization), it is extremely laborious to assure that all samples are analyzed during the log phase of the reaction (for both the target gene and the normalization gene). Another method, quantitative competitive (QC)–PCR, has been developed and is used widely for PCR quantitation. QC–PCR relies on the inclusion of an internal control competitor in each reaction (Becker-Andre 1991; Piatak et al. 1993a,b). The efficiency of each reaction is normalized to the internal competitor. A known amount of internal competitor can be

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added to each sample. To obtain relative quantitation, the unknown target PCR product is compared with the known competitor PCR product. Success of a quantitative competitive PCR assay relies on developing an internal control that amplifies with the same efficiency as the target molecule. The design of the competitor and the validation of amplification efficiencies require a dedicated effort. However, because QC–PCR does not require that PCR products be analyzed during the log phase of the amplification, it is the easier of the two methods to use.

Several detection systems are used for quantitative PCR and RT–PCR analysis: (1) agarose gels, (2) fluorescent labeling of PCR products and detection with laser-induced fluorescence using capillary electrophoresis (Fasco et al. 1995; Williams et al. 1996) or acrylamide gels, and (3) plate capture and sandwich probe hybridization (Mulder et al. 1994). Although these methods proved successful, each method requires post-PCR manipulations that add time to the analysis and may lead to laboratory contamination. The sample throughput of these methods is limited (with the exception of the plate capture approach), and, therefore, these methods are not well suited for uses demanding high sample throughput (i.e., screening of large numbers of biomolecules or analyzing samples for diagnostics or clinical trials).

Here we report the development of a novel assay for quantitative DNA analysis. The assay is based on the use of the 5′ nuclease assay first described by Holland et al. (1991). The method uses the 5′ nuclease activity of *Taq* polymerase to cleave a nonextendible hybridization probe during the extension phase of PCR. The approach uses dual-labeled fluorogenic hybridization probes (Lee et al. 1993; Bassler et al. 1995; Livak et al. 1995a,b). One fluorescent dye serves as a reporter [FAM (i.e., 6-carboxyfluorescein)] and its emission spectra is quenched by the second fluorescent dye, TAMRA (i.e., 6-carboxy-tetramethylrhodamine). The nuclease degradation of the hybridization probe releases the quenching of the FAM fluorescent emission, resulting in an increase in peak fluorescent emission at 518 nm. The use of a sequence detector (ABI Prism) allows measurement of fluorescent spectra of all 96 wells of the thermal cycler continuously during the PCR amplification. Therefore, the reactions are monitored in real time. The output data is described and quantitative analysis of input target DNA sequences is discussed below.

RESULTS

PCR Product Detection in Real Time

The goal was to develop a high-throughput, sensitive, and accurate gene quantitation assay for use in monitoring lipid-mediated therapeutic gene delivery. A plasmid-encoding human factor VIII gene sequence, pF8TM (see Methods), was used as a model therapeutic gene. The assay uses fluorescent Taqman methodology and an instrument capable of measuring fluorescence in real time (ABI Prism 7700 Sequence Detector). The Taqman reaction requires a hybridization probe labeled with two different fluorescent dyes. One dye is a reporter dye (FAM), the other is a quenching dye (TAMRA). When the probe is intact, fluorescent energy transfer occurs and the reporter dye fluorescent emission is absorbed by the quenching dye (TAMRA). During the extension phase of the PCR cycle, the fluorescent hybridization probe is cleaved by the 5′–3′ nucleolytic activity of the DNA polymerase. On cleavage of the probe, the reporter dye emission is no longer transferred efficiently to the quenching dye, resulting in an increase of the reporter dye fluorescent emission spectra. PCR primers and probes were designed for the human factor VIII sequence and human β-actin gene (as described in Methods). Optimization reactions were performed to choose the appropriate probe and magnesium concentrations yielding the highest intensity of reporter fluorescent signal without sacrificing specificity. The instrument uses a charge-coupled device (i.e., CCD camera) for measuring the fluorescent emission spectra from 500 to 650 nm. Each PCR tube was monitored sequentially for 25 msec with continuous monitoring throughout the amplification. Each tube was re-examined every 8.5 sec. Computer software was designed to examine the fluorescent intensity of both the reporter dye (FAM) and the quenching dye (TAMRA). The fluorescent intensity of the quenching dye, TAMRA, changes very little over the course of the PCR amplification (data not shown). Therefore, the intensity of TAMRA dye emission serves as an internal standard with which to normalize the reporter dye (FAM) emission variations. The software calculates a value termed ΔRn (or ΔRQ) using the following equation: $\Delta Rn = (Rn^+) - (Rn^-)$, where Rn^+ = emission intensity of reporter/emission intensity of quencher at any given time in a reaction tube, and Rn^- = emission intensitity of re-

porter/emission intensity of quencher measured prior to PCR amplification in that same reaction tube. For the purpose of quantitation, the last three data points (ΔRns) collected during the extension step for each PCR cycle were analyzed. The nucleolytic degradation of the hybridization probe occurs during the extension phase of PCR, and, therefore, reporter fluorescent emission increases during this time. The three data points were averaged for each PCR cycle and the mean value for each was plotted in an "amplification plot" shown in Figure 1A. The ΔRn mean value is plotted on the *y*-axis, and time, represented by cycle number, is plotted on the *x*-axis. During the early cycles of the PCR amplification, the ΔRn value remains at base line. When sufficient hybridization probe has been cleaved by the *Taq* polymerase nuclease activity, the intensity of reporter fluorescent emission increases. Most PCR amplifications reach a plateau phase of reporter fluorescent emission if the reaction is carried out to high cycle numbers. The amplification plot is examined early in the reaction, at a point that represents the log phase of product accumulation. This is done by assigning an arbitrary threshold that is based on the variability of the base-line data. In Figure 1A, the threshold was set at 10 standard deviations above the mean of base-line emission calculated from cycles 1 to 15. Once the threshold is chosen, the point at which

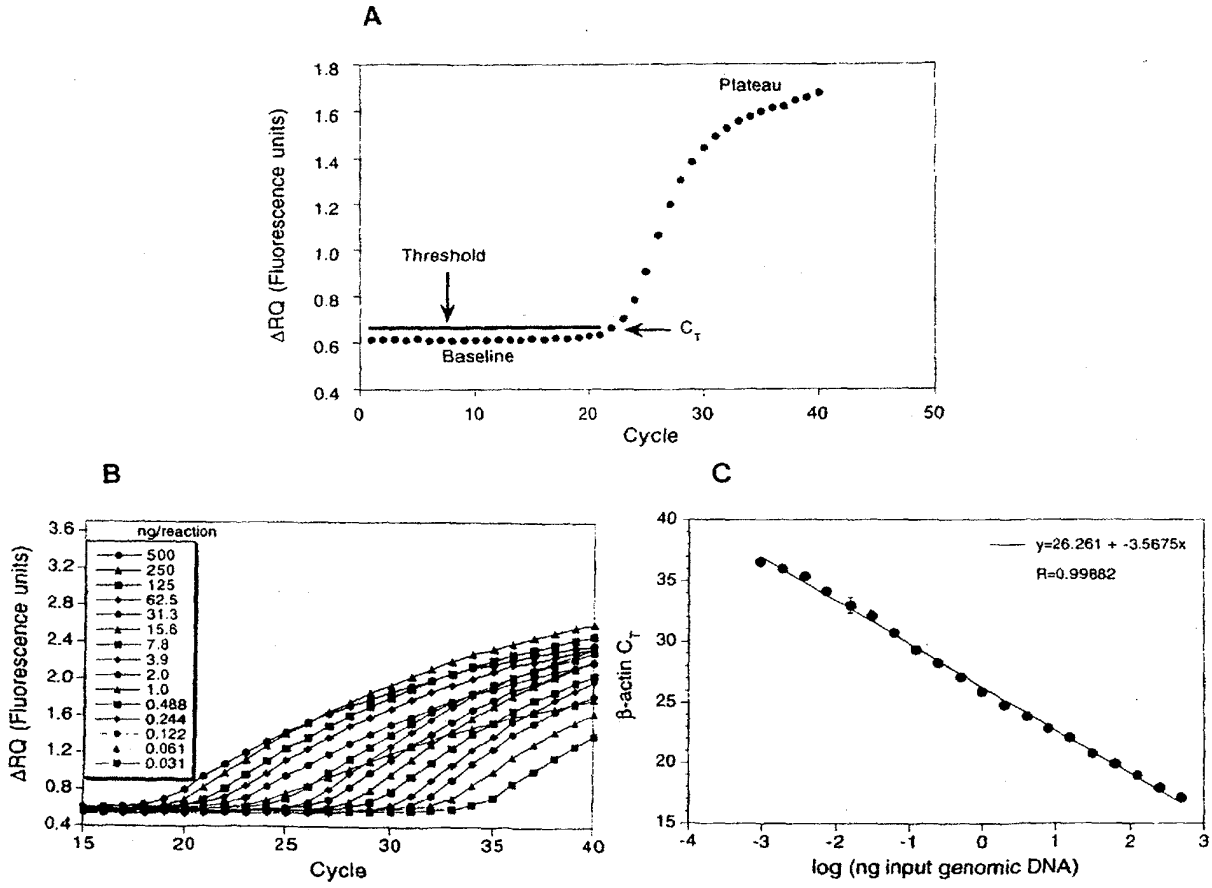

Figure 1 PCR product detection in real time. (*A*) The Model 7700 software will construct amplification plots from the extension phase fluorescent emission data collected during the PCR amplification. The standard deviation is determined from the data points collected from the base line of the amplification plot. C_T values are calculated by determining the point at which the fluorescence exceeds a threshold limit (usually 10 times the standard deviation of the base line). (*B*) Overlay of amplification plots of serially (1:2) diluted human genomic DNA samples amplified with β-actin primers. (C) Input DNA concentration of the samples plotted versus C_T. All points represent the mean of triplicate PCR amplifications, and error bars are shown (but not always visible).

the amplification plot crosses the threshold is defined as C_T. C_T is reported as the cycle number at this point. As will be demonstrated, the C_T value is predictive of the quantity of input target.

C_T Values Provide a Quantitative Measurement of Input Target Sequences

Figure 1B shows amplification plots of 15 different PCR amplifications overlaid. The amplifications were performed on a 1:2 serial dilution of human genomic DNA. The amplified target was human β-actin. The amplification plots shift to the right (to higher threshold cycles) as the input target quantity is reduced. This is expected because reactions with fewer starting copies of the target molecule require greater amplification to degrade enough probe to attain the threshold fluorescence. An arbitrary threshold of 10 standard deviations above the base line was used to determine the C_T values. Figure 1C represents the C_T values plotted versus the sample dilution value. Each dilution was amplified in triplicate PCR amplifications and plotted as mean values with error bars representing one standard deviation. The C_T values decrease linearly with increasing target quantity. Thus, C_T values can be used as a quantitative measurement of the input target number. It should be noted that the amplification plot for the 15.6-ng sample shown in Figure 1B does not reflect the same fluorescent rate of increase exhibited by most of the other samples. The 15.6-ng sample also achieves endpoint plateau at a lower fluorescent value than would be expected based on the input DNA. This phenomenon has been observed occasionally with other samples (data not shown) and may be attributable to late cycle inhibition; this hypothesis is still under investigation. It is important to note that the flattened slope and early plateau do not impact significantly the calculated C_T value as demonstrated by the fit on the line shown in Figure 1C. All triplicate amplifications resulted in very similar C_T values—the standard deviation did not exceed 0.5 for any dilution. This experiment contains a >100,000-fold range of input target molecules. Using C_T values for quantitation permits a much larger assay range than directly using total fluorescent emission intensity for quantitation. The linear range of fluorescent intensity measurement of the ABI Prism 7700 Sequence Detector only spans three logs, resulting in only a 1000-fold dynamic range of input molecules. Thus, C_T values provide accurate measurements over a very large range of relative starting target quantities.

Sample Preparation Validation

Several parameters influence the efficiency of PCR amplification: magnesium and salt concentrations, reaction conditions (i.e., time and temperature), PCR target size and composition, primer sequences, and sample purity. All of the above factors are common to a single PCR assay, except sample to sample purity. In an effort to validate the method of sample preparation for the factor VIII assay, PCR amplification reproducibility and efficiency of 10 replicate sample preparations were examined. After genomic DNA was prepared from the 10 replicate samples, the DNA was quantitated by ultraviolet spectroscopy. Amplifications were performed analyzing β-actin gene content in 100 and 25 ng of total genomic DNA. Each PCR amplification was performed in triplicate. Comparison of C_T values for each triplicate sample show minimal variation based on standard deviation and coefficient of variance (Table 1). Therefore, each of the triplicate PCR amplifications was highly reproducible, demonstrating that real time PCR using this instrumentation introduces minimal variation into the quantitative PCR analysis. Comparison of the mean C_T values of the 10 replicate sample preparations also showed minimal variability, indicating that each sample preparation yielded similar results for β-actin gene quantity. The highest C_T difference between any of the samples was 0.85 and 0.71 for the 100 and 25 ng samples, respectively. Additionally, the amplification of each sample exhibited an equivalent rate of fluorescent emission intensity change per amount of DNA target analyzed as indicated by similar slopes derived from the sample dilutions (Fig. 2). Any sample containing an excess of a PCR inhibitor would exhibit a greater measured β-actin C_T value for a given quantity of DNA. In addition, the inhibitor would be diluted along with the sample in the dilution analysis (Fig. 2), altering the expected C_T value change. Each sample amplification yielded a similar result in the analysis, demonstrating that this method of sample preparation is highly reproducible with regard to sample purity.

Quantitative Analysis of a Plasmid After Transient Transfection

293 cells were transiently transfected with a vec-

Table 1. Reproducibility of Sample Preparation Method

	100 ng				25 ng			
Sample no.	C_T	mean	standard deviation	CV	C_T	mean	standard deviation	CV
1	18.24				20.48			
	18.23				20.55			
	18.33	18.27	0.06	0.32	20.5	20.51	0.03	0.17
2	18.33				20.61			
	18.35				20.59			
	18.44	18.37	0.06	0.32	20.41	20.54	0.11	0.54
3	18.3				20.54			
	18.3				20.6			
	18.42	18.34	0.07	0.36	20.49	20.54	0.06	0.28
4	18.15				20.48			
	18.23				20.44			
	18.32	18.23	0.08	0.46	20.38	20.43	0.05	0.26
5	18.4				20.68			
	18.38				20.87			
	18.46	18.42	0.04	0.23	20.63	20.73	0.13	0.61
6	18.54				21.09			
	18.67				21.04			
	19	18.74	0.24	1.26	21.04	21.06	0.03	0.15
7	18.28				20.67			
	18.36				20.73			
	18.52	18.39	0.12	0.66	20.65	20.68	0.04	0.2
8	18.45				20.98			
	18.7				20.84			
	18.73	18.63	0.16	0.83	20.75	20.86	0.12	0.57
9	18.18				20.46			
	18.34				20.54			
	18.36	18.29	0.1	0.55	20.48	20.51	0.07	0.32
10	18.42				20.79			
	18.57				20.78			
	18.66	18.55	0.12	0.65	20.62	20.73	0.1	0.46
Mean	(1–10)	18.42	0.17	0.90		20.66	0.19	0.94

tor containing a partial cDNA for human factor VIII, pF8TM. A series of transfections was set up using a decreasing amount of the plasmid (40, 4, 0.5, and 0.1 μg). Twenty-four hours post-transfection, total DNA was purified from each flask of cells. β-Actin gene quantity was chosen as a value for normalization of genomic DNA concentration from each sample. In this experiment, β-actin gene content should remain constant relative to total genomic DNA. Figure 3 shows the result of the β-actin DNA measurement (100 ng total DNA determined by ultraviolet spectroscopy) of each sample. Each sample was analyzed in triplicate and the mean β-actin C_T values of the triplicates were plotted (error bars represent one standard deviation). The highest difference between any two sample means was 0.95 C_T. Ten nanograms of total DNA of each sample were also examined for β-actin. The results again showed that very similar amounts of genomic DNA were present; the maximum mean β-actin C_T value difference was 1.0. As Figure 3 shows, the rate of β-actin C_T change between the 100- and 10-ng samples was similar (slope values range between −3.56 and −3.45). This verifies again that the method of sample preparation yields samples of identical PCR integrity (i.e., no sample contained an excessive amount of a PCR inhibitor). However, these results indicate that each sample contained slight differences in the actual amount of genomic DNA analyzed. Determination of actual genomic DNA concentration was accomplished

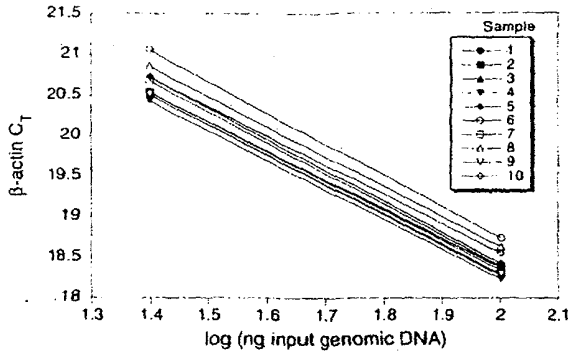

Figure 2 Sample preparation purity. The replicate samples shown in Table 1 were also amplified in tripicate using 25 ng of each DNA sample. The figure shows the input DNA concentration (100 and 25 ng) vs. C_T. In the figure, the 100 and 25 ng points for each sample are connected by a line.

by plotting the mean β-actin C_T value obtained for each 100-ng sample on a β-actin standard curve (shown in Fig. 4C). The actual genomic DNA concentration of each sample, *a*, was obtained by extrapolation to the *x*-axis.

Figure 4A shows the measured (i.e., non-normalized) quantities of factor VIII plasmid DNA (pF8TM) from each of the four transient cell transfections. Each reaction contained 100 ng of total sample DNA (as determined by UV spectroscopy). Each sample was analyzed in triplicate

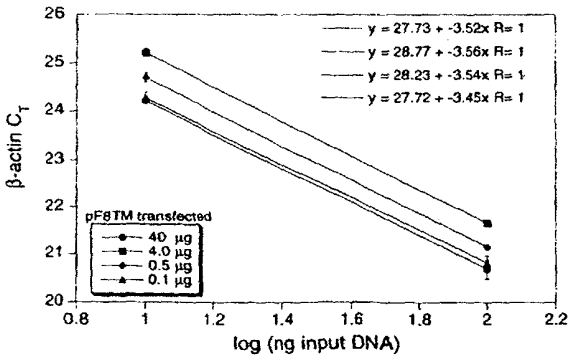

Figure 3 Analysis of transfected cell DNA quantity and purity. The DNA preparations of the four 293 cell transfections (40, 4, 0.5, and 0.1 µg of pF8TM) were analyzed for the β-actin gene. 100 and 10 ng (determined by ultraviolet spectroscopy) of each sample were amplified in triplicate. For each amount of pF8TM that was transfected, the β-actin C_T values are plotted versus the total input DNA concentration.

PCR amplifications. As shown, pF8TM purified from the 293 cells decreases (mean C_T values increase) with decreasing amounts of plasmid transfected. The mean C_T values obtained for pF8TM in Figure 4A were plotted on a standard curve comprised of serially diluted pF8TM, shown in Figure 4B. The quantity of pF8TM, *b*, found in each of the four transfections was determined by extrapolation to the *x*-axis of the standard curve in Figure 4B. These uncorrected values, *b*, for pF8TM were normalized to determine the actual amount of pF8TM found per 100 ng of genomic DNA by using the equation:

$$\frac{b \times 100\text{ ng}}{a} = \begin{array}{l}\text{actual pF8TM copies per}\\ \text{100 ng of genomic DNA}\end{array}$$

where *a* = actual genomic DNA in a sample and *b* = pF8TM copies from the standard curve. The normalized quantity of pF8TM per 100 ng of genomic DNA for each of the four transfections is shown in Figure 4D. These results show that the quantity of factor VIII plasmid associated with the 293 cells, 24 hr after transfection, decreases with decreasing plasmid concentration used in the transfection. The quantity of pF8TM associated with 293 cells, after transfection with 40 µg of plasmid, was 35 pg per 100 ng genomic DNA. This results in ~520 plasmid copies per cell.

DISCUSSION

We have described a new method for quantitating gene copy numbers using real-time analysis of PCR amplifications. Real-time PCR is compatible with either of the two PCR (RT–PCR) approaches: (1) quantitative competitive where an internal competitor for each target sequence is used for normalization (data not shown) or (2) quantitative comparative PCR using a normalization gene contained within the sample (i.e., β-actin) or a "housekeeping" gene for RT–PCR. If equal amounts of nucleic acid are analyzed for each sample and if the amplification efficiency before quantitative analysis is identical for each sample, the internal control (normalization gene or competitor) should give equal signals for all samples.

The real-time PCR method offers several advantages over the other two methods currently employed (see the introduction). First, the real-time PCR method is performed in a closed-tube system and requires no post-PCR manipulation

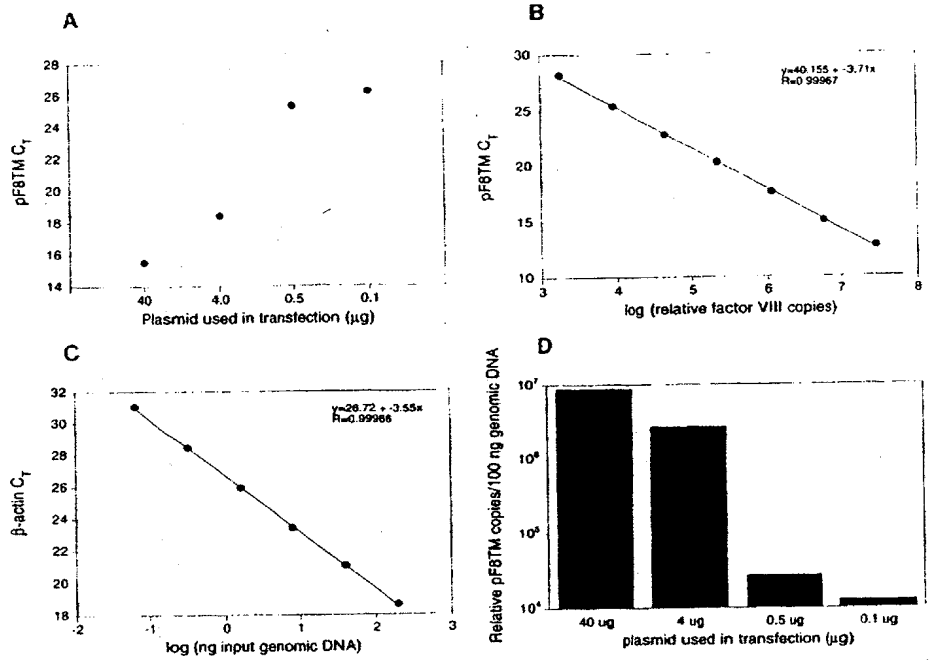

Figure 4 Quantitative analysis of pF8TM in transfected cells. (*A*) Amount of plasmid DNA used for the transfection plotted against the mean C_T value determined for pF8TM remaining 24 hr after transfection. (*B,C*) Standard curves of pF8TM and β-actin, respectively. pF8TM DNA (*B*) and genomic DNA (*C*) were diluted serially 1:5 before amplification with the appropriate primers. The β-actin standard curve was used to normalize the results of *A* to 100 ng of genomic DNA. (*D*) The amount of pF8TM present per 100 ng of genomic DNA.

of sample. Therefore, the potential for PCR contamination in the laboratory is reduced because amplified products can be analyzed and disposed of without opening the reaction tubes. Second, this method supports the use of a normalization gene (i.e., β-actin) for quantitative PCR or housekeeping genes for quantitative RT–PCR controls. Analysis is performed in real time during the log phase of product accumulation. Analysis during log phase permits many different genes (over a wide input target range) to be analyzed simultaneously, without concern of reaching reaction plateau at different cycles. This will make multigene analysis assays much easier to develop, because individual internal competitors will not be needed for each gene under analysis. Third, sample throughput will increase dramatically with the new method because there is no post-PCR processing time. Additionally, working in a 96-well format is highly compatible with automation technology.

The real-time PCR method is highly reproducible. Replicate amplifications can be analyzed for each sample minimizing potential error. The system allows for a very large assay dynamic range (approaching 1,000,000-fold starting target). Using a standard curve for the target of interest, relative copy number values can be determined for any unknown sample. Fluorescent threshold values, C_T, correlate linearly with relative DNA copy numbers. Real time quantitative RT–PCR methodology (Gibson et al., this issue) has also been developed. Finally, real time quantitative PCR methodology can be used to develop high-throughput screening assays for a variety of applications [quantitative gene expression (RT–PCR), gene copy assays (Her2, HIV, etc.), genotyping (knockout mouse analysis), and immuno-PCR].

Real-time PCR may also be performed using intercalating dyes (Higuchi et al. 1992) such as ethidium bromide. The fluorogenic probe method offers a major advantage over intercalating dyes—greater specificity (i.e., primer dimers and nonspecific PCR products are not detected).

METHODS

Generation of a Plasmid Containing a Partial cDNA for Human Factor VIII

Total RNA was harvested (RNAzol B from Tel-Test, Inc., Friendswood, TX) from cells transfected with a factor VIII expression vector, pCIS2.8c25D (Eaton et al. 1986; Gorman et al. 1990). A factor VIII partial cDNA sequence was generated by RT-PCR [GeneAmp EZ tTth RNA PCR Kit (part N808-0179, PE Applied Biosystems, Foster City, CA)] using the PCR primers F8for and F8rev (primer sequences are shown below). The amplicon was reamplified using modified F8for and F8rev primers (appended with *Bam*HI and *Hin*dIII restriction site sequences at the 5′ end) and cloned into pGEM-3Z (Promega Corp., Madison, WI). The resulting clone, pF8TM, was used for transient transfection of 293 cells.

Amplification of Target DNA and Detection of Amplicon Factor VIII Plasmid DNA

(pF8TM) was amplified with the primers F8for 5′-CCCGTGCCAAGAGTGACGTGTC-3′ and F8rev 5′-AAACCTCAGCCTGGATGGTAGG-3′. The reaction produced a 422-bp PCR product. The forward primer was designed to recognize a unique sequence found in the 5′ untranslated region of the parent pCIS2.8c25D plasmid and therefore does not recognize and amplify the human factor VIII gene. Primers were chosen with the assistance of the computer program Oligo 4.0 (National Biosciences, Inc., Plymouth, MN). The human β-actin gene was amplified with the primers β-actin forward primer 5′-TCACCCACACTGTGCCCATCTACGA-3′ and β-actin reverse primer 5′-CAGCGGAACCGCTCATTGCCAATGG-3′. The reaction produced a 295-bp PCR product.

Amplification reactions (50 μl) contained a DNA sample, 10× PCR Buffer II (5 μl), 200 μM dATP, dCTP, dGTP, and 400 μM dUTP, 4 mM $MgCl_2$, 1.25 Units AmpliTaq DNA polymerase, 0.5 unit AmpErase uracil *N*-glycosylase (UNG), 50 pmole of each factor VIII primer, and 15 pmole of each β-actin primer. The reactions also contained one of the following detection probes (100 nM each): F8probe 5′(FAM)AGCTCTCCACCTGCTTCTTTCTGTGCCTT(TAMRA)p-3′ and β-actin probe 5′-(FAM)ATGCCCX(TAMRA)CCCCCATGCCATCp-3′ where p indicates phosphorylation and X indicates a linker arm nucleotide. Reaction tubes were MicroAmp Optical Tubes (part number N801-0933, Perkin Elmer) that were frosted (at Perkin Elmer) to prevent light from reflecting. Tube caps were similar to MicroAmp Caps but specially designed to prevent light scattering. All of the PCR consumables were supplied by PE Applied Biosystems (Foster City, CA) except the factor VIII primers, which were synthesized at Genentech, Inc. (South San Francisco, CA). Probes were designed using the Oligo 4.0 software, following guidelines suggested in the Model 7700 Sequence Detector instrument manual. Briefly, probe T_m should be at least 5°C higher than the annealing temperature used during thermal cycling; primers should not form stable duplexes with the probe.

The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C. Thermal cycling proceeded with 40 cycles of 95°C for 0.5 min and 60°C for 2 min. All reactions were performed in the Model 7700 Sequence Detector (PE Applied Biosystems), which contains a GeneAmp PCR System 9600. Reaction conditions were programmed on a Power Macintosh 7100 (Apple Computer, Santa Clara, CA) linked directly to the Model 7700 Sequence Detector. Analysis of data was also performed on the Macintosh computer. Collection and analysis software was developed at PE Applied Biosystems.

Transfection of Cells with Factor VIII Construct

Four T175 flasks of 293 cells (ATCC CRL 1573), a human fetal kidney suspension cell line, were grown to 80% confluency and transfected pF8TM. Cells were grown in the following media: 50% HAM'S F12 without GHT, 50% low glucose Dulbecco's modified Eagle medium (DMEM) without glycine with sodium bicarbonate, 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin–streptomycin. The media was changed 30 min before the transfection. pF8TM DNA amounts of 40, 4, 0.5, and 0.1 μg were added to 1.5 ml of a solution containing 0.125 M $CaCl_2$ and 1× HEPES. The four mixtures were left at room temperature for 10 min and then added dropwise to the cells. The flasks were incubated at 37°C and 5% CO_2 for 24 hr, washed with PBS, and resuspended in PBS. The resuspended cells were divided into aliquots and DNA was extracted immediately using the QIAamp Blood Kit (Qiagen, Chatsworth, CA). DNA was eluted into 200 μl of 20 mM Tris-HCl at pH 8.0.

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(54) Oligonucleotides and method for detecting bacteria.

(57) A synthetic oligonucleotide which is complementary to a nucleotide sequence of a gene selected from the group consisting of the *Shiga* toxin gene of *Shigella* species, the *ipa*H gene of *Shigella* species and EIEC, the *inv*E gene of *Shigella* species and EIEC, the *ara*C gene of *Salmonella* species, the Verocytotoxin-1 gene of EHEC or VTEC, the Verocytotoxin-2 gene of EHEC or VTEC, the toxic shock syndrome toxin-1 gene of *Staphylococcus aureus*, the *ctx* gene of *Vibrio cholerae*, and the enterotoxin gene of *Clostridium perfringens*; a method for detecting a bacterial strain by amplifying a region of the above gene by PCR using the above oligonucleotides as primers and detecting the amplified region; and a kit for the detection of the bacterial strain.

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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to detection of pathogenic bacteria in samples (e.g., clinical isolates and food specimens) for the purposes of diagnoses, screenings, quarantine inspections, and clinical tests. Specifically, it relates to detection of pathogens associated with bacterial food poisoning and bacterial diarrhea. More specifically, it relates to detection of enteropathogenic bacteria including *Shigella* species, *Salmonella* species, enterohemorrhagic *Escherichia coli* or Verocytotoxin-producing *Escherichia coli*, *Staphylococcus aureus*, *Vibrio chlolerae*, and *Clostridium perfringens*.

Discussion of the Related Art

Detection of pathogenic bacteria such as *Shigella* species, *Salmonella* species, enterohemorrhagic *Escherichia coli* (hereinafter simply referred to as EHEC) or Verocytotoxin-producing *Escherichia coli* - (hereinafter simply referred to as VTEC), *Staphylococcus aureus*, *Vibrio cholerae*, and *Clostridium perfringens* is an important task in the field of medicine and public hygiene, and various methods have been used.

Conventionally, detection of a pathogenic bacterial strain involves isolation of several pathogenic bacterial colonies and identification of the species of the bacteria by serological or biochemical method.

In the case of *Shigella* species, this has been achieved by culturing and isolating the target bacterium from specimens of patient stools, food, or the like, using a medium, such as DHL agar or MacConkey's agar, and then further culturing the bacterium using a medium such as TSI agar or LIM agar for the purpose of identification.

In the case of *Salmonella* species, culture is conducted for isolation of the bacteria from specimens of patient stools or vomits, food or wiping samples, etc., followed by inoculation to TSI agar, SIM medium, VP-MR medium and lysine decarboxylation test medium and subsequent overnight culture at 37 °C, to confirm *Salmonella* species, and the serotype is determined using a commercially available set of antisera against O and H antigens.

EHEC or VTEC has been found to cause hemolytic uremic syndrome in children, as well as food poisoning symptoms, typically hemorrhagic colitis, and stress has recently been placed on detection of this bacterium in clinical tests. In the case of detecting EHEC or VTEC, specimens are patient stools, food, or water samples (drinking water, river water, etc.) collected from the environment surrounding the patient. In detecting EHEC (VTEC) in these specimens, it is necessary to perform a series of procedures from direct isolation culture, a primary confirmation culture test, and a secondary confirmation culture test to an agglutination test with an antiserum.

In the case of *Staphylococcus aureus*, specimens are patient vomits or stools, food the patient ate, samples wiped out from the environment surrounding the patient, or the like. Before *Staphylococcus aureus* is detected and identified in these specimens, it is necessary to perform bacterial culture, isolation culture and then pure culture and confirmation culture.

In the case of *Vibrio cholerae*, specimens are patient stools or food, or water samples (drinking water, river water, sea water, etc.) or benthos samples collected from the environment surrounding the patient. In detecting and identifying *Vibrio cholerae* in these specimens, it is necessary to perform a series of procedures from primary enrichment culture, secondary enrichment culture, and isolation culture to an agglutination reaction test with anti-*V. cholerae* O1 serum and confirmation culture.

In the case of *Clostridium perfringens*, specimens are obtained mainly from patient stools and food. For detection and identification, the specimens are subjected to enrichment culture and isolation culture under anaerobic conditions. With several colonies of the bacteria, tests for biochemical properties are conducted.

Any identification process mentioned above usually takes several days, and hampers rapid diagnoses of infectious diseases.

Specifically, in the case of *Shigella* species, each culture step takes 18-24 hours, totalling 3-4 days; rapid detection is difficult. Other available methods include the reversed passive latex agglutination using a specific antibody to the Shiga toxin, the EIA method using a specific antibody to the 140 MDal plasmid product associated with the pathogenicity of *Shigella* species and enteroinvasive *Escherichia coli* - [Kenichiro Ito et al., Japanese Journal of Bacteriology 41, 414 (1986)] and the DNA probe method for detecting the *ipa*B gene, the *ipa*C gene, or the *ipa*D gene (US Patent Application No. 888194). However, these testing methods require complicated troublesome procedures in preparing reagents and specimens,

and take much time.

In the case of *Salmonella* species, 2-3 days are taken for bacterial isolation and identification of the bacteria from specimens. In addition, *Salmonella* tests are difficult to conduct in ordinary laboratories, because as many as 100 antisera and much experience are required to achieve complete serum typing of *Salmonella* species, which involve a large number of serum types. Also, each culture step and serotyping test take 3-4 days; rapidity is poor. Moreover, confirmation culture and serotyping are expensive and involve troublesome operation.

In the case of EHEC (VTEC), each culture step takes 18-24 hours, totalling as many as 3-4 days. The currently representative serotype of EHEC (VTEC) is 0157:H7, but no diagnostic antiserum has been commercially available for identification of this serotype, so that the diagnostic antiserum has to be prepared by the investigator. In addition, it is often difficult to identify the causative bacterium solely on the basis of serum typing in EHEC (VTEC), because the serum type and the pathogenicity do not always agree with each other. Therefore, the conventional testing method for EHEC (VTEC) lacks rapidity and simplicity, and is not suitable for practical application.

In the case of *Staphylococcus aureus*, each culture step takes 18-24 hours, totalling as many as about 4 days when combined with the time required for the subsequent testings. Also, in the biochemical test in culture for identification, various properties, such as aerobic growth, VP reactivity, nitrate reduction, Tween 80 hydrolyzability, hyaluronidase activity and sugar decomposition, should be examined, but this process is troublesome, tedious and expensive. The most reliable method for identifying the causative bacterium for food poisoning and diarrhea is to test the isolated strain for exotoxin (toxic shock syndrome toxin -1, hereinafter simply referred to as TSST-1) production. However, even when a commercially available convenient reagent kit is used, 18-20 hours will be taken to obtain the results; rapidity is poor.

In the case of *Vibrio cholerae*, each culture step takes 18-24 hours, totalling as many as about 4 days. In the biochemical test concerning confirmation culture, various properties, such as oxidase test positivity, indole test positivity, motility, and lysine decarboxylation test positivity should be examined. These tests are troublesome, tedious and expensive, and the results obtained are difficult to assess in some cases. Moreover, in the case of *Vibrio cholerae*, it is essential to test the isolated strain for enterotoxin (cholera toxin; CT) production to take an administrative measure for pest control. However, even when a commercially available convenient reagent kit is used, 18-20 hours will be taken to obtain the results; rapidity is poor and practical applicability is low.

In the case of Welch's bacillus(*Clostridium perfringens*), the detection requires considerably long time: each culture step takes 18-48 hours, totalling 5-6 days. In addition, since *Clostridium perfringens* strains are widely distributed in the nature, only the detection of the bacterial strain from specimens is not enough to determine the strain as the causative agent for food poisoning. Further tests are required, including detection of the enterotoxin in patient stool, assay of the isolated strain for enterotoxin production, serotype determination, and bacterial count for suspected food. These procedures consume much time and labor, and lack rapidity and simplicity.

In recent years, the DNA probing or hybridization using oligonucleotides has been tried. However, when hybridization is performed on a membrane or on other supports using a probe of a labeled oligonucleotide, followed by detection of the probe, sensitivity of the assays depends on numbers of organisms available for detection. Therefore it is difficult to achieve a high detection sensitivity and selectivity in this test without the above-described pretreatment of the separation culture.

SUMMARY OF THE INVENTION

It is object of the present invention to provide synthetic oligonucleotides used as primers for PCR to amplify certain regions of the genes specific to the above various pathogenic microorganisums.

It is another object of the present invention to provide a simple, rapid and highly sensitive process for detecting the above various pathogenic microorganisms for quarantine inspection, clinical laboratory examination and food inspection, wherein a region of a gene specific to the bacterial strain to be detected is amplified by the PCR technique using synthetic oligonucleotide primers.

It is still another object of the present invention to provide a kit for detection of the above various bacterial strains, comprising at least a pair of primers, a thermostable DNA polymerase, and dNTP solutions.

The gist of the present invention relates to:

1) A synthetic oligonucleotide of 10 to 30 bases which is complementary to a nucleotide sequence of a gene selected from the group consisting of the *Shiga* toxin gene of *Shigella* species, the *ipa*H gene of *Shigella* species and enteroinvasive *Escherichia coli* (hereinafter simply referred to as EIEC), the *inv*E

gene of *Shigella* species and EIEC, the *ara*C gene of *Salmonella* species, the Verocytotoxin-1 gene of EHEC or VTEC, the Verocytotoxin-2 gene of EHEC or VTEC, the toxic shock syndrome toxin gene of *Staphylococcus aureus*, the *ctx* gene of *Vibrio cholerae*, and the enterotoxin gene of *Clostridium perfringens*;

2) A synthetic oligonucleotide comprising a nucleotide sequence complementary to the synthetic oligonucleotide of 1);

3) A method for detecting a bacterial strain selected from the group consisting of *Shigella* species, EIEC, *Salmonella* species, EHEC, VTEC, *Staphylococcus aureus*, *Vibrio cholerae* and *Clostridium perfringens*, wherein the method comprises:

(1) hybridizing one primer to a single-stranded target DNA as a template DNA present in a specimen and carrying out a primer extension reaction to give a primer extension product,

(2) denaturing the resulting DNA duplex to separate the primer extension product from the template DNA; the primer extension product functioning as the other template DNA for the other primer,

(3) repeating a cycle of simultaneous primer extension reaction with the two primers, separation of the primer extension products from the templates, and hybridization of primers to amplify a region of the target DNA, in the steps from (1) to (3), the primers being selected from the group consisting of oligonucleotides of 1) and 2),

(4) detecting the amplified nucleotide sequence to determine whether a suspected bacterial strain is present in the specimen; and

4) A kit for detection of a bacterial strain, comprising at least a pair of primers selected from the group consisting of oligonucleotides of 1) and 2), a thermostable DNA polymerase, and dNTP solutions.

The present invention provides a highly selective and highly sensitive method for rapid detection of *Shigella* species having the Shiga toxin gene, the *ipa*H gene and the *inv*E gene, EIEC having the *ipa*H gene and the *inv*E gene, *Salmonella* species having the *ara*C gene, EHEC having the VT1 gene and the VT2 gene, *Staphylococcus aureus* having the TSST-1 gene, Vibrio cholerae having the *ctx* gene, and *Clostridium perfringens* having the enterotoxin gene.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given hereinbelow and the accompanying drawings which are given by way of illustration only, and thus, are not limitative of the present invention.

Figure 1 is the pattern of the electrophoresis of the amplified DNA fragments on an agarose gel to evaluate the sensitivity of the detection method of the present invention for the TSST-1 gene of *Staphylococcus aureus*, wherein numerals 1 to 9 refer to the number of DNA copies used in the polymerase chain reaction (hereinafter simply referred to as PCR): 1 means 10^7 copies; 2, 10^6 copies; 3, 10^5 copies; 4, 10^4 copies; 5, 10^3 copies; 6, 10^2 copies; 7, 10 copies; 8, 1 copy; and 9, no DNA.

Figure 2 is the pattern of the agarose gel electrophoresis of the nucleotide fragments amplified by PCR to evaluate the specificity of the detection method of the present invention for the *ctx* gene of *Vibrio cholerae*, wherein M indicates the molecular weight marker and lanes 1-13 indicate the template DNA solutions containing heat extract of the following strains:

Lanes 1 to 3: *Vibrio cholerae* (El Tor - Ogawa type, the *ctx* gene positive strain)
Lanes 4 to 6: *Vibrio cholerae* (El Tor - Inaba type, the *ctx* gene positive strain)
Lane 7: *Vibrio cholerae* (Classical- Ogawa type, the *ctx* gene positive strain)
Lane 8: *Vibrio cholerae* (Classical- Inaba type, the *ctx* gene positive strain)
Lanes 9 to 10: *Vibrio cholerae* (non-O1, the *ctx* gene positive strain)
Lane 11: *Vibrio cholerae* (El Tor - Ogawa type, the *ctx* gene negative strain)
Lane 12: *Vibrio cholerae* (El Tor - Inaba type, the *ctx* gene negative strain)
Lane 13: Enterotoxigenic *Escherichia coli*(Thermolabile enterotoxin gene positive strain).

Figure 3 is the electrophoretic pattern of the agarose gel electrophoresis for the nucleotide fragments amplified by PCR to detect the enterotoxin gene of *Clostridium perfringens*, the upper part being the results obtained with Oligonucleotide SEQ ID NO:28 + Oligonucleotide SEQ ID NO:33; the lower part being the results obtained with Oligonucleotide SEQ ID NO:29 + Oligonucleotide SEQ ID NO:33, wherein M indicates the molecular weight marker and lanes 1-13 indicate the template DNA derived from heat extracts of the following strains: Lane 1, ATCC 12925; Lane 2, ATCC 12924; Lane 3, ATCC 12922; Lane 4, ATCC 12920; Lane 5, ATCC 12916; Lane 6, ATCC 12915; Lane 7, ATCC 12918; Lane 8, ATCC 12919; Lane 9, ATCC 12921; Lane 10, JCM 1296; Lane 11, JCM 1416; Lane 12, JCM 1382; and Lane 13, negative control.

Figure 4 shows the results of a Southern blot hybridisation test to confirm if the nucleotide sequence of amplified DNA with the primers of the present invention is a part of the enterotoxin gene sequences of *Clostridium perfringens*, the upper part being the results obtained with Oligonucleotide SEQ ID NO:28 + Oligonucleotide SEQ ID NO:33; the lower part being the results obtained with Oligonucleotide SEQ ID NO:29 + Oligonucleotide SEQ ID NO:33, wherein M indicates the molecular weight marker and lanes 1-13 indicate the template DNA solutions derived from heat extracts of the following strains: Lane 1, ATCC 12925; Lane 2, ATCC 12924; Lane 3, ATCC 12922; Lane 4, ATCC 12920; Lane 5, ATCC 12916; Lane 6, ATCC 12915; Lane 7, ATCC 12918; Lane 8, ATCC 12919; Lane 9, ATCC 12921; Lane 10, JCM 1296; Lane 11, JCM 1416; Lane 12, JCM 1382; and Lane 13, negative control.

DETAILED DESCRIPTION OF THE INVENTION

Oligonucleotides

An oligonucleotide of the present invention is a synthetic oligonucleotide which complementarily and selectively hybridizes to a gene specific to a pathogenic bacterial strain such as *Shigella* species, EIEC, *Salmonella* species, EHEC or VTEC, *Stapbylococcus aureus, Vibrio cholerae*, and *Clostridium perfringens*. In the bacterial detection of the present invention, the oligonucleotides are used as primers in the PCR to amplify a DNA sequence of a target gene specific to the pathogenic bacteria to be detected. For this purpose, any combination of two oligonucleotides of the present invention may optionally be employed without particular limitation as long as a DNA sequence of 50 to 2000 bases, preferably of 100 to 1000 bases, can be amplified. The preferred combination of oligonucleotides are described in detail in the following preferred embodiments.

In the oligonucleotides mentioned below, T may be replaced with U without impairing the advantageous effect of the oligonucleotide.

Preferred Embodiment 1:

For the detection of *Shigella species* (*Shigella* dysenteriae, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*) and EIEC, the Shiga toxin gene specific to *Shigella* species, the *ipa*H gene specific to *Shigella* species and EIEC, or the *inv*E gene specific to *Shigella* species and EIEC is selected as the target gene.

When the Shiga toxin gene is targeted, two oligonucleotides, one comprising at least 10 consecutive bases of the following oligonucleotide SEQ ID No.1 and the other comprising at least 10 consecutive bases of the following oligonucleotide SEQ ID NO: 2, are selected in the present invention:

(5')-CAACACTGGATGATCTCAG-(3') (SEQ ID NO: 1)

(5')-CCCCCTCAACTGCTAATA-(3') (SEQ ID NO: 2)

When the *ipa*H gene is targeted, two oligonucleotides, one comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:3 and the other comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:4 are selected in the present invention:

(5')-TGTATCACAGATATGGCATGC-(3') (SEQ ID NO:3)

(5')-TCCGGAGATTGTTCCATGTG-(3') (SEQ ID NO:4)

When the *inv*E gene is targeted, two oligonucleotides, one comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:5 and the other comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:6 are selected in the present invention:

(5')-CAAGATTTAACCTTCGTCAACC-(3') (SEQ ID NO:5)

(5')-AGTTCTCGGATGCTATGCTC-(3') (SEQ ID NO:6)

Preferred Embodiment 2:

For the detection of *Salmonella* species, the *ara*C gene is targeted.

For this purpose, any one of the following oligonucleotide combinations is preferably selected in the present invention:

a combination in which one oligonucleotide comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:7 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:8; a combitnation in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:9 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:10; and a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO: 11 and the other

comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO: 8:

(5')-CGCGGAGAGGGCGTCATT-(3') (SEQ ID NO:7)
(5')-GCAACGACTCATTAATTACCG-(3') (SEQ ID NO:8)
(5')-ATCTGGTCGCCGGGCTGA-(3') (SEQ ID NO:9)
(5')-GCATCGCGCACACGGCTA-(3') (SEQ ID NO:10)
(5')-GGCGAGCAGTTTGTCTGTC-(3') (SEQ ID NO:11)

Preferred Embodiment 3:

For the detection of EHEC or VTEC strains, the Verocytotoxin-1 (VT1) gene or the Verocytotoxin-2 (VT2) gene is targeted.

In order to detect bacteria which have only the VT1 gene, two oligonucleotides, one comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:12 and the other comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:13, are preferably selected in the present invention:

(5')-CAACACTGGATGATCTCAG-(3') (SEQ ID NO:12)
(5')-CCCCCTCAACTGCTAATA-(3') (SEQ ID NO:13)

In order to detect bacteria which have only the VT2 gene or its variant genes (VT2vha, VT2vhb and VT2vp1), two oligonucleotides, one comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:14 and the other comprising at least 10 consecutive bases of oligonucleotide SEQ ID NO:15, are preferably selected in the present invention:

(5')-ATCAGTCGTCACTCACTGGT-(3') (SEQ ID NO:14)
(5')-CCAGTTATCTGACATTCTG-(3') (SEQ ID NO:15)

In order to detect bacteria which have both the VT1 gene and the VT2 gene (including the VT2vha, VT2vhb and VT2vp1 genes), any one of the following oligonucleotide combinations is preferably selected in the present invention:

a combination in which one oligonucleotide comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:16 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:18; and a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:17 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:18:

(5')-AGTTTACGTTAGACTTTTCGAC-(3') (SEQ ID NO:16)
(5')-CGGACAGTAGTTATACCAC-(3') (SEQ ID NO:17)
(5')-CTGCTGTCACAGTGACAAA-(3') (SEQ ID NO:18)

Preferred Embodiment 4:

For the detection of *Staphylococcus aureus*, the TSST-1 gene is targeted.

For this purpose, any one of the following oligonucleotide combination is preferably selected in the present invention: a combination in which one oligonucleotide comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:20 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:21; a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:19 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:22; and a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO: 20 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO: 22:

(5')-CCTTTAAAAGTTAAGGTTCATG-(3') (SEQ ID NO:19)
(5')-GGCCAAAGTTCGATAAAAAAC-(3') (SEQ ID NO:20)
(5')-ATTTATAGGTGGTTTTTCAGTAT-(3') (SEQ ID NO:21)
(5')-CTGCTTCTATAGTTTTTATTTCA-(3') (SEQ ID NO:22)

Preferred Embodiment 5:

For the detection of *Vibrio chlolerae*, the *ctx* gene is targeted.

For this purpose, any one of the following oligonucleotide combinations is preferably selected in the present invention:

a combination in which one oligonucleotide comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:23 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:25; and a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:24 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:26:

(5')-TGATGAAATAAAGCAGTCAGGT-(3') (SEQ ID NO:23)

(5')-ACAGAGTGAGTACTTTGACC-(3') (SEQ ID NO:24)
(5')-GGCACTTCTCAAACTAATTGAG-(3') (SEQ ID NO:25)
(5')-ATACCATCCATATATTTGGGAG-(3') (SEQ ID NO:26)

Preferred Embodiment 6:

For the detection of *Clostridium perfringens*, the enterotoxin gene is targeted.

For this purpose, any one of the following oligonucleotide combinations is preferably selected in the present invention:

a combination in which one oligonucleotide comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:27 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:32; a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:28 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:33; a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:29 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:33; a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:30 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:34; and a combination in which one comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:31 and the other comprises at least 10 consecutive bases of oligonucleotide SEQ ID NO:35:

(5')-TCTGAGGATTTAAAAACACC-(3') (SEQ ID NO:27)
(5')-ACCCTCAGTAGGTTCAAGTC-(3') (SEQ ID NO:28)
(5')-ATGAAACAGGTACCTTTAGCC-(3') (SEQ ID NO:29)
(5')-GGTAATATCTCTGATGATGGAT-(3') (SEQ ID NO:30)
(5')-TAACTCATACCCTTGGACTC-(3') (SEQ ID NO:31)
(5')-GAACCTTGATCAATATTTCC-(3') (SEQ ID NO:32)
(5')-GTAGCAGCAGCTAAATCAAGG-(3') (SEQ ID NO:33)
(5')-AGTCCAAGGGTATGAGTTAG-(3') (SEQ ID NO:34)
(5')-CCATCACCTAAGGACTGTTC-(3') (SEQ ID NO:35)

Amplification of gene sequence by PCR

For amplification of a region of a target gene in the present invention, the PCR developed by Saiki et al. [Science 230, 1350 (1985)] is employed.

Specifically, two oligonucleotide primers that flank a specific region of a target gene (in the present invention, the Shiga toxin gene of *Shigella* species, the *ipa*H and *inv*E genes of *Shigella* species and EIEC, the *ara*C gene of *Salmonella* species, the VT1 and VT2 genes of EHEC or VTEC, the TSST-1 gene of *Staphylococcus aureus*, the *ctx* gene of *Vibrio cholerae* and the enterotoxin gene of *Clostridium perfringens*) are synthesized.

In PCR, one of the oligonucleotide primers selectively hybridizes to the (+)-strand of a target gene DNA, and the other hybridizes to the (-)-strand of the DNA. Then, both the oligonucleotides serve as primers of template dependent DNA polymerization respectively. In the present invention, single strand DNAs formed by heat denaturation of double strand DNAs in specimens are used as templates. The duplexes resulting from the DNA polymerization reaction are then denatured to separate the primer extension products from the templates. Then, the primer extension products themselves serve as the templates for the next DNA polymerization reaction. The cycle of denaturation, primer annealing in which a primer hybridizes with a template DNA and a primer extension reaction is repeated until the region of the target gene is amplified enough for its detection.

Specimens applicable to the PCR in the present invention may include clinical samples such as stool, urine, blood, tissue homogenate, and food samples. A specimen for PCR should be pre-treated to release the nucleic acid components from the bacterial cells present therein. Since PCR can be carried out with only several to several tens of nucleic acid molecules, a test solution containing an adequate amount of nucleic acid can be prepared simply by treating a specimen with a bacteriolytic enzyme, a surfactant or an alkali for a short time.

Oligonucleotides used as primers in the present invention may be either synthetic or natural, and in view of selectivity, detection sensitivity and reproducibility, they are not less than 10 bases in length, preferably not less than 15 bases. It is not necessary to label the primers for detection.

The region to be amplified in a target gene (i.e., the Shiga toxin gene of *Shigella* species, the *ipa*H gene and the *inv*E gene of EIEC, the VT1 gene and the VT2 gene of EHEC or VTEC, the *ara*C gene of

Salmonella species, the TSST-1 gene of *Staphylococcus aureus*, and the *ctx* gene of Vibrio cholerae, and the enterotoxin gene of *Clostridium perfringens*) is 50 to 2000 bases in length, preferably 100 to 1000 bases.

In PCR, a thermostable DNA polymerase is used. The origins from which the enzyme is derived are not particularly limited as long as the enzyme maintains its activity at a temperature of from 90 to 95 °C. The denaturation is carried out at a temperature of from 90 to 95 °C, the primer annealing from 37 to 65 °C, and the polymerization reaction from 50 to 75 °C. The cycle of denaturation, primer annealing and polymerization is repeated for 20 to 42 cycles.

The presence or absence, and the length of the amplified nucleotide fragment can be detected by subjecting the reaction solution to agarose gel electrophoresis after the completion of PCR. Other types of electrophoresis and chromatography can also be used for the detection. One of the oligonucleotide primers may be used as a probe to detect the amplified nucleotide sequence.

The detection of a nucleotide sequence of a target gene in a specimen means that the bacterial strain having the gene is present in the specimen.

The invention will now be described in more detail by the following examples, but it should be noted that the invention is not limited to these examples.

EXAMPLES

Example 1: Detection of *Shigella* species having the Shiga toxin gene

[Experiment 1]

Preparation of specimens

The 42 strains of *Shigella dysenteriae* listed in Table 1 are obtained from patients or other sources. Each strain is inoculated to LB medium (1%- tryptone, 0.5% yeast extract, 1% sodium chloride), and subjected to overnight shaking culture at 37 °C under aerobic conditions. Each culture broth is diluted 10 folds with 10 mM Tris-HCl buffer, pH 7.5 (hereinafter referred to as TE buffer), and heated at 95 °C for 10 minutes, followed by centrifugation; the supernatants are used as specimen solutions.

Synthesis of primers

As primers for amplifying the Shiga toxin gene of *Shigella dysenteriae*, the above-described oligonucleotides SEQ ID NO:1 and SEQ ID NO:2 are selected based upon the known base sequence of the Shiga toxin gene [Takao, T. et al., Microb. Pathog., 5:357-369 (1988)], and chemically synthesized by the β-cyanoethylphosphoamidite method using a Cyclone Plus DNA synthesizer (produced by MilliGen/Bioresearch). The synthesized oligonucleotides are purified by high performance liquid chromatography using a C18 reversed-phase column.

The Shiga toxin gene is regarded as identical to the VT1 gene of EHEC or VTEC, with difference only in several bases [Takao, T. et al., Microb. Pathog., 5:357-369 (1988)].

PCR

To 3 μl of the above-described specimen solution, 17.05 μl of sterile distilled water, 3 μl of 10 x reaction buffer, 4.8 μl of dNTP solution, 1.0 μl of primer (1), 1.0 μl of primer (2), and 0.15 μl of a thermostable DNA polymerase are added to prepare 30 μl of a reaction mixture. This reaction mixture is overlaid with 50 μl of mineral oil (produced by SIGMA). The contents of the solutions used and the primers (1) and (2) are as follows:

10 x reaction buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.1% (w/v) gelatin.

dNTP solution: A mixture of dATP, dCTP, dGTP and dTTP, each having a final concentration of 1.25 mM.

Primers (1) and (2): Aqueous solution of the above-described chemically synthesized purified products (concentration, 3.75 OD/ml).

Primers: The above-described chemically synthesized and purified products are used in combination as follows:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:1 + Oligonucleotide SEQ ID NO:2

Thermostable DNA polymerase: Taq DNA polymerase (5 unit/ml; produced by Perkin Elmer Cetus).

The reaction conditions are as follows:

Thermal denaturation: 94 °C for 1 minute.

Annealing: 55 °C for 1 minute.

Polymerization: 72 °C for 1 minute.

The cycle of thermal denaturation, primer annealing and polymerization (5.7 minutes) is repeated for 35 cycles (entire time, about 3 hours). This procedure is performed using a DNA thermal cycler (produced by Perkin Elmer Cetus) in which the above reaction conditions are programmed.

Detection

Agarose gel electrophoresis

To detect the amplified nucleotide fragment in the reaction mixture, agarose gel electrophoresis is conducted as mentioned below.

The agarose gel used has a gel concentration of 3% (w/v) and contains ethidium bromide (0.5 µl/ml). Electrophoresis is performed at the constant voltage of 100 V for 30 minutes. Operation procedures and other conditions described by Maniatis et al.[Molecular Cloning, 2nd edition (1989)] are used. In addition to the reaction mixture, molecular weight markers are also electrophoresed concurrently. The length of the nucleotide fragment is calculated by comparing the relative mobilities.

Reversed passive latex agglutination (RPLA) test

A commercially available RPLA kit for detection of *Escherichia coli* Verocytotoxin (produced by DENKA SEIKEN) is purchased. Specimens are prepared and tested according to the instruction manual attached.

Results

The base sequence of the Shiga toxin gene of *Shigella dysenteriae* has already been determined. Therefore, the length of the nucleotide amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when the oligonucleotides SEQ ID NO: 1 and SEQ ID NO: 2 of the present invention are used in combination, a nucleotide sequence of 349 bases (or a nucleotide duplex of 349 base pairs) is amplified. When this estimation accords with the length of the amplified nucleotide fragment, it is judged that PCR using the combination of primers accurately amplify the target region of the Shiga toxin gene, and that the bacterial strain in the specimen has the Shiga toxin gene. The results obtained from the agarose gel electrophoresis with 34 test strains are given in Table 1. PCR using the primers of the present invention amplifies only the DNA of the strains which give positive results in the RPLA, showing no amplification of DNAs of Shiga toxin negative strains. This indicates that PCR using the primers of the present invention are capable of accurately amplifying the Shiga toxin gene and that *Shigella dysenteriae* having the Shiga toxin gene can be detected with high accuracy by using the oligonucleotides of the present invention.

Table 1

No	Strains		RPLA	PCR	No	Strains		RPLA	PCR
01	S. dysenteriae	TUMD 1	-	-	22	S. dysenteriae	AQ7370	-	-
02	S. dysenteriae	TUMD 2	-	-	23	S. dysenteriae	AQ7403	-	-
03	S. dysenteriae	TUMD 3	-	-	24	S. dysenteriae	AA-22021	+	+
04	S. dysenteriae	TUMD 4	-	-	25	S. dysenteriae	AA-22184	+	+
05	S. dysenteriae	TUMD 5	-	-	26	S. dysenteriae	AA-22192	+	+
06	S. dysenteriae	TUMD 6	-	-	27	S. dysenteriae	AA-22555	+	+
07	S. dysenteriae	MARABLA	-	-	28	S. dysenteriae	AA-21933	+	+
08	S. dysenteriae	AQ7003	+	+	29	S. dysenteriae	AA-22496	+	+
09	S. dysenteriae	AQ7004	+	+	30	S. dysenteriae	AA-22224	+	+
10	S. dysenteriae	AQ7018	-	-	31	S. dysenteriae	AA-22542	+	+
11	S. dysenteriae	AQ7029	-	-	32	S. dysenteriae	AA-22616	+	+
12	S. dysenteriae	AQ7030	-	-	33	S. dysenteriae	AA-22239	+	+
13	S. dysenteriae	AQ7061	-	-	34	S. dysenteriae	AA-22538	+	+
14	S. dysenteriae	AQ7125	-	-	35	S. dysenteriae	ATCC9361	+	+
15	S. dysenteriae	AQ7131	-	-	36	S. dysenteriae	ATCC9753	-	-
16	S. dysenteriae	AQ7151	-	-	37	S. dysenteriae	ATCC9764	-	-
17	S. dysenteriae	AQ7164	-	-	38	S. dysenteriae	ATCC11456a	+	+
18	S. dysenteriae	AQ7166	-	-	38	S. dysenteriae	ATCC13313	+	+
19	S. dysenteriae	AQ7234	-	-	40	S. dysenteriae	ATCC23351	+	+
20	S. dysenteriae	AQ7302	-	-	41	S. dysenteriae	ATCC29027	-	-
21	S. dysenteriae	AQ7350	-	-	42	S. dysenteriae	ATCC29028	-	-

Note)
+ : DNA of estimated length is amplified.
N : DNA of not-estimated length is amplified.
-: DNA is not amplified.

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to the Shiga toxin gene, the DNAs of clinically important pathogenic bacteria other than *Shigella dysenteriae* are examined with the primers of the present invention. The same procedure as used in Experiment 1 is followed, except for the method of preparation of specimens.

Preparation of specimens

Each strain listed in Table 2 is inoculated to an appropriate enrichment medium, and subjected to overnight culture at 37 °C under aerobic or anaerobic conditions (*Clostridium perfringens*, *Campylobacter jejuni*, *Campylobacter coli*, *Bacteroides flagilis*, *Bacteroides vulgatus*, *Lactobacillus acidophilus*, and *Bifidobacterium adolescentis* are cultured under anaerobic conditions, while *Neisseria gonorrhoeae* and *Neisseria meningitidis* are cultured in the presence of 3 - 10% CO_2). Bacterial cells are centrifugally recovered from 0.5 ml of each culture broth, and once washed with TE buffer. To these bacterial cells, an N-acetylmuraminidase solution in 50 mM phosphate buffer, pH 7.5, and an achromopeptidase solution in

the same buffer are added to final concentrations of 50 μg/ml and 1 mg/ml, respectively, followed by incubation at 37°C for 10 minutes to lyse the cells. A 1:1 phenol/chloroform mixture, saturated with TE buffer, is added to the lysate, followed by vigorous stirring. After centrifugation, the supernatant is recovered, and treated with ethanol to precipitate the nucleic acids. The resulting precipitate is dissolved in 1 ml of TE buffer; this solution is used as a specimen. Also, Human placenta DNA, at a concentration of 1 μg/ml, is subjected to PCR in the same manner as above.

Results

Table 2 shows the results of the test using the combination of the primers of the present invention. This combination of primers does not amplify DNAs other than those of Shiga toxin-producing *Shigella dysenteriae* and Verocytotoxin-1-producing *Escherichia coli*. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with DNAs of the bacteria having the Shiga toxin gene.

The agarose gel electrophoresis used in the above examples of the present invention can differentiate nucleotide fragments from one another which are different in length by 5-10 bases (base pairs) for nucleotide fragments of not more than 100 bases (base pairs), and by 10-20 bases (base pairs) for nucleotide fragments of 100-500 bases (base pairs). In addition, the use of other gel material such as acrylamide makes it possible to improve the precision in measuring the length of nucleotide fragment. Thus, the reliability of the selective detection of the target gene in the present invention can further be increased.

Table 2

No	Strains		PCR	No	Strains		PCR
01	Bacillus cereus	ATCC14579	-	21	Klebsiella pneumoniae	JCM1662	-
02	Bacillus Subtillis	JCM1465	-	22	Proteus vulgaris	JCM1668	-
03	Staphylococcus aureus	JCM2413	-	23	Citrobacter freundii	ATCC33128	-
04	Staphylococcus epidermidis	JCM2414	-	24	Streptococcus pyogenes	ATCC12344	-
05	Salmonella typhimurium	IFO12529	-	25	Streptococcus pneumoniae	ATCC33400	-
06	Salmonella enteritidis	IFO3163	-	26	Elaemophilis influenzae	ATCC33391	-
07	Clostridium perfringens	ATCC12917	-	27	Proteus mirabilis	ATCC29906	-
08	Vibrio cholerae	ATCC25872	-	28	Neisseria meningitidis	ATCC13077	-
09	Vibrio cholerae type Ogawa	ATCC9458	-	29	Neisseria gonorrhoeae	ATCC19424	-
10	Vibrio cholerae type Inaba	ATCC9459	-	30	Listeria monocytogenes	ATCC15313	-
11	Vibrio fluvialis	JCM3752	-	31	Lactobacillus acidophilus	JCM1132	-
12	Campylobacter jejuni	JCM2013	-	32	Bifidobacterium adolescentis	JCM1275	-
13	Campylobacter coli	JCM2529	-	33	Fusobacterium nucleatum	ATCC25586	-
14	Escherichia coli	JCM1649	-	34	Propionibacterium acnes	ATCC6919	-
15	Yersinia enterocolitica	ATCC9610	-	35	Veillonella atypica	ATCC17744	-
16	Shigella flexneri	ATCC29903	-	36	Pseudomonas aeruginosa	IFO12689	-
17	Shigella sonnei	ATCC29930	-	37	Corynebacterium diphtheriae	JCM1310	-
18	Bacteroides flagilis	ATCC23745	-	38	Peptostreptococcus anaerobius	ATCC27337	-
19	Bacteroides vulgatus	JCM5826	-	39	Human piacental	DNA	-
20	Enterococcus faecalis	JCM5803	-				

Note)
+ : DNA of estimated length is amplified.
N : DNA of not-estimated length is amplified.
- : DNA is not amplified.

Example 2: Detection of *Shigella* species and EIEC both having the *ipa*H gene

[Experiment 1]

Preparation of specimens

The same procedure as used in Example 1 is followed except that 341 strains of *Shigella* species and EIEC listed in Tables 3-1 to 3-7 are used.

Synthesis of primers

As primers for amplifying the *ipa*H gene of *Shigella* species and EIEC strains, the above-described oligonucleotides SEQ ID NO:3 and SEQ ID NO:4 are selected based upon the known base sequence of the ipaH gene [Hartman, A.B., et al., J. Bacteriol., 172, 1905-1915(1990); Venkatesan, M.M., et al., Mol. Microbiol., 5, 2435-2446 (1991)]. These oligonucleotides are chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that the following oligonucleotide combination is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:3 + Oligonucleotide SEQ ID NO:4

Detection

Agarose gel electrophoresis

The same procedure as in Example 1 is followed.

Colony hybridization test

A colony hybridization test is carried out using an oligonucleotide probe specific to the *ipa*H gene according to the procedure described by Grunstein [Grunstein, M. and Hogness, D., Proc. Natl. Acad. Sci., 72, 3961(1975)].

Results

The base sequence of the *ipa*H gene of *Shigella* species and EIEC has already been determined. Therefore, the length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when the oligonucleotides SEQ ID NO: 3 and SEQ ID NO: 4 of the present invention are used in combination, a nucleotide fragment of 242 bases (or a nucleotide duplex of 242 base pairs) should be amplified. When this estimation accords with the length of the amplified nucleotide fragment, it is judged that PCR using the combination of primers accurately amplify the target region in the *ipa*H gene, and that the bacterial strain in the specimen has the *ipa*H gene. The results obtained from the agarose gel electrophoresis with 341 test strains are given in Tables 3-1 to 3-7. PCR using the primers of the present invention amplifies only the DNA of the strains which give the *ipa*H positive results in the colony hybridization test, showing no amplification of the DNAs of *ipa*H negative strains. This indicates that PCR using the primers of the present invention is capable of accurately amplifying the *ipa*H gene and that *Shigella* species and EIEC both having the *ipa*H gene can be detected with high accuracy by using the oligonucleotides of the present invention.

Table 3 — 1

No	Strains	CH test*	Primer 3+4 **	No	Strains	CH test*	Primer 3+4 **
001	S. dysenteriae TUMD 1	+	+	026	S. dysenteriae AA-22192	+	—
002	S. dysenteriae TUMD 2	+	+	027	S. dysenteriae AA-22555	+	—
003	S. dysenteriae TUMD 3	+	+	028	S. dysenteriae AA-21933	+	+
004	S. dysenteriae TUMD 4	+	+	029	S. dysenteriae AA-22496	+	+
005	S. dysenteriae TUMD 5	+	+	030	S. dysenteriae AA-22224	+	+
006	S. dysenteriae TUMD 6	+	+	031	S. dysenteriae AA-22542	+	+
007	S. dysenteriae MARABIA	—	—	032	S. dysenteriae AA-22616	—	+
008	S. dysenteriae AQ-7003	+	+	033	S. dysenteriae AA-22239	+	+
009	S. dysenteriae AQ-7004	+	+	034	S. dysenteriae AA-22538	+	+
010	S. dysenteriae AQ-7018	+	+	035	S. dysenteriae ATCC9361	+	+
011	S. dysenteriae AQ-7029	+	+	036	S. dysenteriae ATCC9753	+	+
012	S. dysenteriae AQ-7030	+	+	037	S. dysenteriae ATCC11456a	+	+
013	S. dysenteriae AQ-7061	+	+	038	S. dysenteriae ATCC13313	+	+
014	S. dysenteriae AQ-7125	+	+	039	S. dysenteriae ATCC23351	+	+
015	S. dysenteriae AQ-7131	+	+	040	S. dysenteriae ATCC29027	+	+
016	S. dysenteriae AQ-7151	+	+	041	S. dysenteriae ATCC29028	+	+
017	S. dysenteriae AQ-7164	+	+	042	S. flexneri TUMD 7	+	+
018	S. dysenteriae AQ-7166	+	+	043	S. flexneri TUMD 8	+	+
019	S. dysenteriae AQ-7234	+	+	044	S. flexneri TUMD 9	+	+
020	S. dysenteriae AQ-7302	+	+	045	S. flexneri TUMD10	+	+
021	S. dysenteriae AQ-7350	+	+	046	S. flexneri TUMD11	+	+
022	S. dysenteriae AQ-7370	+	+	047	S. flexneri TUMD12	+	+
023	S. dysenteriae AQ-7403	+	+	048	S. flexneri TUMD13	+	+
024	S. dysenteriae AA-22021	+	+	049	S. flexneri TUMD14	+	+
025	S. dysenteriae AA-22184	+	+	050	S. flexneri TUMD15	+	+

Note) * Colony hybridization test
** Numerals refer to SEQ ID NOs.

Table 3 – 2

No	Strains	CH test*	Primer 3+4 **	No	Strains	CH test*	Primer 3+4 **
051	S. flexneri TUMD16	+	+	076	S. flexneri TUMD42	+	+
052	S. flexneri TUMD17	+	+	077	S. flexneri TUMD43	+	+
053	S. flexneri TUMD18	+	+	078	S. flexneri TUMD44	+	+
054	S. flexneri TUMD19	+	+	079	S. flexneri TUMD45	+	+
055	S. flexneri TUMD20	+	+	080	S. flexneri TUMD46	+	+
056	S. flexneri TUMD21	+	+	081	S. flexneri TUMD47	+	+
057	S. flexneri TUMD22	+	+	082	S. flexneri TUMD48	+	+
058	S. flexneri TUMD23	+	+	083	S. flexneri TUMD49	+	+
059	S. flexneri TUMD24	+	+	084	S. flexneri TUMD50	+	+
060	S. flexneri TUMD25	+	+	085	S. flexneri TUMD51	+	+
061	S. flexneri TUMD26	+	+	086	S. flexneri TUMD52	+	+
062	S. flexneri TUMD27	+	+	087	S. flexneri TUMD53	+	+
063	S. flexneri TUMD28	+	+	088	S. flexneri TUMD54	+	+
064	S. flexneri TUMD29	+	+	089	S. flexneri TUMD55	+	+
065	S. flexneri TUMD30	+	+	090	S. flexneri TUMD56	+	+
066	S. flexneri TUMD31	+	+	091	S. flexneri TUMD57	+	+
067	S. flexneri TUMD32	+	+	092	S. flexneri TUMD58	+	+
068	S. flexneri TUMD33	+	+	093	S. flexneri TUMD59	+	+
069	S. flexneri TUMD34	+	+	094	S. flexneri TUMD60	+	+
070	S. flexneri TUMD35	+	+	095	S. flexneri TUMD61	+	+
071	S. flexneri TUMD36	+	+	096	S. flexneri Maramba 89-77	+	+
072	S. flexneri TUMD38	+	+	097	S. flexneri Maramba 89-95	+	+
073	S. flexneri TUMD39	+	+	098	S. flexneri Maramba89-109a	+	+
074	S. flexneri TUMD40	+	+	099	S. flexneri Maramba89-119	+	+
075	S. flexneri TUMD41	+	+	100	S. flexneri Maramba89-155	+	+

Table 3 – 3

No	Strains	CH test*	Primer 3+4 **	No	Strains	CH test*	Primer 3+4 **
101	S. flexneri Maramba89-164	+	+	126	S. flexneri AQ-7385	+	+
102	S. flexneri Maramba89-150	+	+	127	S. flexneri AQ-7386	+	+
103	S. flexneri AA-22175	+	+	128	S. flexneri AQ-7390	+	+
104	S. flexneri AA-22371	+	+	129	S. flexneri AQ-7391	+	+
105	S. flexneri AA-22266	+	+	130	S. flexneri AQ-7393	+	+
106	S. flexneri AA-22636	+	+	131	S. flexneri AQ-7394	+	+
107	S. flexneri AA-22187	+	+	132	S. flexneri AQ-7398	+	+
108	S. flexneri AA-22170	+	+	133	S. flexneri AQ-7399	+	+
109	S. flexneri AA-22367	+	+	134	S. flexneri AQ-7400	+	+
110	S. flexneri AA-22316	+	+	135	S. flexneri AQ-7402	+	+
111	S. flexneri AA-22265	+	+	136	S. flexneri AQ-7407	+	+
112	S. flexneri AA-22296	+	+	137	S. flexneri AQ-7408	+	+
113	S. flexneri AA-22312	+	+	138	S. flexneri AQ-7411	+	+
114	S. flexneri AA-22246	+	+	139	S. flexneri AQ-7416	+	+
115	S. flexneri AA-21981	+	+	140	S. flexneri AQ-7417	+	+
116	S. flexneri AA-22097	+	+	141	S. flexneri AQ-7418	+	+
117	S. flexneri AQ-7347	+	+	142	S. flexneri AQ-7423	+	+
118	S. flexneri AQ-7348	+	+	143	S. flexneri AQ-7424	+	+
119	S. flexneri AQ-7351	+	+	144	S. flexneri AQ-7426	+	+
120	S. flexneri AQ-7360	+	+	145	S. flexneri AQ-7427	+	+
121	S. flexneri AQ-7367	+	+	146	S. flexneri Manila 89-164	+	–
122	S. flexneri AQ-7372	+	+	147	S. flexneri Manila 89-177	+	+
123	S. flexneri AQ-7378	+	+	148	S. flexneri Manila 89-209	+	+
124	S. flexneri AQ-7379	+	+	149	S. flexneri Manila 89-210	+	+
125	S. flexneri AQ-7380	+	+	150	S. flexneri Manila 89-229	+	+

Table 3 — 4

No	Strains	CH test*	Primer 3+4 **	No	Strains	CH test*	Primer 3+4 **
151	S. flexneri Manila 89-230	−	−	176	S. boydii TUMD64	+	+
152	S. flexneri Manila 89-231	+	+	177	S. boydii TUMD65	+	+
153	S. flexneri Manila 89-232	+	+	178	S. boydii TUMD66	+	+
154	S. flexneri Manila 89-233	+	+	179	S. boydii TUMD67	+	+
155	S. flexneri Manila 89-273	+	+	180	S. boydii TUMD68	+	+
156	S. flexneri Manila 89-328	+	+	181	S. boydii AQ-7019	+	+
157	S. flexneri Manila 89-333	+	+	182	S. boydii AQ-7020	+	+
158	S. flexneri Manila 89-365	+	+	183	S. boydii AQ-7026	+	+
159	S. flexneri Manila 89-274	+	+	184	S. boydii AQ-7032	+	+
160	S. flexneri Manila 89-436	+	+	185	S. boydii AQ-7039	+	+
161	S. flexneri Manila 89-438	+	+	186	S. boydii AQ-7042	+	+
162	S. flexneri Manila 89-443	+	+	187	S. boydii AQ-7062	+	+
163	S. flexneri Manila 89-444	+	+	188	S. boydii AQ-7076	+	+
164	S. flexneri Manila 89-450	+	+	189	S. boydii AQ-7098	+	+
165	S. flexneri Manila 89-480	+	+	190	S. boydii AQ-7157	+	+
166	S. flexneri Manila 89-483	+	+	191	S. boydii AQ-7193	+	+
167	S. flexneri Manila 89-486	+	+	192	S. boydii AQ-7206	+	+
168	S. flexneri Manila 89-498	+	+	193	S. boydii AQ-7213	+	+
169	S. flexneri Manila 89-499	−	−	194	S. boydii AQ-7218	+	+
170	S. flexneri Manila 89-503	+	+	195	S. boydii AQ-7238	+	+
171	S. flexneri Manila 89-509	+	+	196	S. boydii AQ-7267	+	+
172	S. flexneri Manila 89-532	+	+	197	S. boydii AQ-7268	+	+
173	S. flexneri Manila 89-539	−	−	198	S. boydii AQ-7307	+	+
174	S. boydii TUMD62	+	+	199	S. boydii AQ-7313	+	+
175	S. boydii TUMD63	+	+	200	S. boydii AQ-7314	+	+

Table 3 – 5

No	Strains		CH test*	Primer 3+4 **	No	Strains		CH test*	Primer 3+4 **
201	S. boydii	AQ-7324	–	–	226	S. sonnei	AQ-7382	+	+
202	S. boydii	AQ-7349	+	+	227	S. sonnei	AQ-7383	+	+
203	S. boydii	AQ-7354	+	+	228	S. sonnei	AQ-7384	+	+
204	S. boydii	AQ-7356	+	+	229	S. sonnei	AQ-7387	+	+
205	S. boydii	AQ-7357	+	+	230	S. sonnei	AQ-7388	+	+
206	S. boydii	AQ-7368	+	+	231	S. sonnei	AQ-7389	+	+
207	S. boydii	AQ-7373	+	+	232	S. sonnei	AQ-7392	+	+
208	S. boydii	AQ-7376	+	+	233	S. sonnei	AQ-7395	+	+
209	S. boydii	AQ-7405	+	+	234	S. sonnei	AQ-7396	+	+
210	S. boydii	AA-22562	+	+	235	S. sonnei	AQ-7397	+	+
211	S. boydii	AA-22241	–	–	236	S. sonnei	AQ-7401	+	+
212	S. boydii	AA-22610	–	–	237	S. sonnei	AQ-7406	+	+
213	S. boydii	AA-20255	+	+	238	S. sonnei	AQ-7409	+	+
214	S. boydii	AA-20211	+	+	239	S. sonnei	AQ-7410	+	+
215	S. boydii	AA-21713	+	+	240	S. sonnei	AQ-7412	+	+
216	S. boydii	AA-17405	–	–	241	S. sonnei	AQ-7413	+	+
217	S. boydii	AA-22804	+	+	242	S. sonnei	AQ-7414	+	+
218	S. boydii	AQ-7297	+	+	243	S. sonnei	AQ-7415	+	+
219	S. sonnei	AQ-7366	+	+	244	S. sonnei	AQ-7419	+	+
220	S. sonnei	AQ-7369	+	+	245	S. sonnei	AQ-7420	+	+
221	S. sonnei	AQ-7371	+	+	246	S. sonnei	AQ-7421	+	+
222	S. sonnei	AQ-7374	+	+	247	S. sonnei	AQ-7422	+	+
223	S. sonnei	AQ-7375	+	+	248	S. sonnei	AQ-7425	+	+
224	S. sonnei	AQ-7377	+	+	249	S. sonnei	AA-22634	+	+
225	S. sonnei	AQ-7381	+	+	250	S. sonnei	AA-22677	+	+

Table 3 – 6

No	Strains		CH test*	Primer 3+4 **	No	Strains		CH test*	Primer 3+4 **
251	S. sonnei	AA-18306	+	+	276	S. sonnei	TUMD91	+	+
252	S. sonnei	AA-22067	+	+	277	S. sonnei	TUMD92	+	–
253	S. sonnei	AA-22870	+	+	278	S. sonnei	TUMD93	+	+
254	S. sonnei	TUMD69	+	+	279	S. sonnei	TUMD94	+	+
255	S. sonnei	TUMD70	+	+	280	S. sonnei	TUMD95	+	+
256	S. sonnei	TUMD71	+	+	281	S. sonnei	TUMD96	+	+
257	S. sonnei	TUMD72	+	+	282	S. sonnei	TUMD97	+	+
258	S. sonnei	TUMD73	+	+	283	S. sonnei	TUMD98	+	+
259	S. sonnei	TUMD74	+	+	284	S. sonnei	TUMD99	+	+
260	S. sonnei	TUMD75	+	+	285	S. sonnei	TUMD100	+	+
261	S. sonnei	TUMD76	+	+	286	S. sonnei	TUMD101	+	+
262	S. sonnei	TUMD77	+	+	287	S. sonnei	TUMD102	+	+
263	S. sonnei	TUMD78	+	+	288	S. sonnei	TUMD103	+	+
264	S. sonnei	TUMD79	+	+	289	S. sonnei	TUMD104	+	+
265	S. sonnei	TUMD80	+	+	290	S. sonnei	TUMD105	+	+
266	S. sonnei	TUMD81	+	+	291	S. sonnei	TUMD106	+	+
267	S. sonnei	TUMD82	+	+	292	S. sonnei	TUMD107	+	+
268	S. sonnei	TUMD83	+	+	293	S. sonnei	TUMD108	+	+
269	S. sonnei	TUMD84	+	+	294	S. sonnei	TUMD109	+	+
270	S. sonnei	TUMD85	+	+	295	S. sonnei	TUMD110	+	+
271	S. sonnei	TUMD86	+	+	296	S. sonnei	TUMD111	+	+
272	S. sonnei	TUMD87	+	+	297	S. sonnei	TUMD112	+	+
273	S. sonnei	TUMD88	+	+	298	S. sonnei	TUMD113	+	+
274	S. sonnei	TUMD89	+	+	299	S. sonnei	TUMD114	+	+
275	S. sonnei	TUMD90	+	+	200	S. sonnei	TUMD115	+	+

Table 3 – 7

No	Strains	CH test*	Primer 3+4 **	No	Strains	CH test*	Primer 3+4 **
301	S. sonnei TUMD116	+	+	326	E. coli AQ8011	+	+
302	S. sonnei TUMD117	+	+	327	E. coli AQ8012	+	+
303	S. sonnei TUMD118	+	+	328	E. coli AQ8013	+	+
304	S. sonnei TUMD119	+	+	329	E. coli AQ8016	+	+
305	S. sonnei TUMD120	+	+	330	E. coli AQ8019	+	+
306	S. sonnei TUMD121	+	+	331	E. coli AQ8022	+	+
307	S. sonnei TUMD122	+	+	332	E. coli AQ8024	+	+
308	S. sonnei TUMD123	+	+	333	E. coli AQ8025	+	+
309	S. sonnei TUMD124	+	+	334	E. coli AQ8027	+	+
310	S. sonnei TUMD125	+	+	335	E. coli AQ8028	+	+
311	S. sonnei TUMD126	+	+	336	E. coli AQ8029	+	+
312	S. sonnei TUMD127	+	+	337	E. coli AQ8031	+	+
313	S. sonnei TUMD128	+	+	338	E. coli AQ8033	–	–
314	S. sonnei Maramba 89-154	+	+	339	E. coli AQ8036	–	–
315	S. sonnei Maramba 89-161	+	+	340	E. coli AQ8044	+	+
316	S. sonnei Manila 89-342	+	+	341	E. coli PE660	+	+
317	S. sonnei Manila 89-445	+	+				
318	E. coli DMR 6	+	+				
319	E. coli DMR78	+	+				
320	E. coli DMR79	+	+				
321	E. coli AQ8001	+	+				
322	E. coli AQ8003	+	+				
323	E. coli AQ8004	+	+				
324	E. coli AQ8008	+	+				
325	E. coli AQ8010	+	+				

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to the *ipa*H gene, the DNAs of clinically important pathogenic bacteria other than *Shigella* species and EIEC are examined with the

primers of the present invention. The same procedure as used in Experiment 1 is followed, except for the procedure of preparation of specimens.

Preparation of specimens

Each strain listed in Table 4 is treated in the same manner as in Experiment 2 of Example 1.

Results

Table 4 shows the results of the test using the combination of primers of the present invention. This combination of primers does not amplify any DNAs other than those of *Shigella* species and EIEC. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with DNAs of the bacteria having the *ipa*H gene.

Table 4

No	Strains		PCR	No	Strains		PCR
01	Bacilius cereus	ATCC14579	-	21	Klebsiella pneumoniae	JCM1662	-
02	Bacillus Subtilis	JCM1465	-	22	Proteus vulgaris	JCM1668	-
03	Staphylococcus aureus	JCM2413	-	23	Citrobacter freundii	ATCC33128	-
04	Staphylococcus epidermidis	JCM2414	-	24	Streptococcus pyogenes	ATCC12344	-
05	Salmonella typhimurium	IFO12529	-	25	Streptococcus pneumoniae	ATCC33400	-
06	Salmonella enteritidis	IFO3163	-	26	Elaemophilis influenzae	ATCC33391	-
07	Clostridium perfringens	ATCC12917	-	27	Proteins mirabilis	ATCC29906	-
08	Vibrio cholerae	ATCC25872	-	28	Neisseria meningitidis	ATCC13077	-
09	Vibrio cholerae type Ogawa	ATCC9458	-	29	Neisseria gonorrhoeae	ATCC19424	-
10	Vibrio cholerae type Inaba	ATCC9459	-	30	Listeria monocytogenes	ATCC15313	-
11	Vibrio fluvialis	JCM3752	-	31	Lactobacillus acidophilus	JCM1132	-
12	Campylobacter jejuni	JCM2013	-	32	Bifidobacterium adolescentis	JCM1275	-
13	Campylobacter coli	JCM2529	-	33	Fusobacterium nucleatum	ATCC25586	-
14	Escherichia coli	JCM1649	-	34	Propionibacterium acnes	ATCC6919	-
15	Yersinia enterocolitica diphtheriae	ATCC9610	-	35	Veillonella atypica	ATCC17744	-
16	Corynebacterium diphtheriae	JCM13	-	36	Pseudomonas aeruginosa	IFO12689	-
17	Peptostreptococcus anaerobius	ATCC23745	-	37	Human placental	DNA	-
18	Bacteroides flagilis	ATCC23745	-				
19	Bacteroides vulgatus	JCM5826	-				
20	Enterococcus faecalis	JCM5803	-				

Example 3: Detection of *Shigella* species and EIEC having the *inv*E gene

[Experiment 1]

Preparation of specimens

The same procedure as used in Example 1 is followed except that 341 strains of *Shigella* species and EIEC listed in Tables 3-1 to 3-7 are used.

Synthesis of primers

As primers for amplifying the *inv*E gene of *Shigella* species and EIEC, the above-described oligonucleotides SEQ ID NO:5 and SEQ ID NO:6 are selected based upon the known base sequence of the *inv*E gene [Watanabe, H., et al., J. Bacteriol., 172, 619-629(1990)]. These oligonucleotides are chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that the following oligonucleotide combination is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:5 + Oligonucleotide SEQ ID NO:6

Detection

Agarose gel electrophoresis

The same procedure as in Example 1 is followed.

Colony hybridization test

A colony hybridization test is carried out using an oligonucleotide probe specific to *inv*E gene according to the procedure described by Grunstein [Grunstein, M. and Hogness, D., Proc. Natl. Acad. Sci., 72, 3961-(1975)].

Results

The base sequence of the *inv*E gene of *Shigella* species and EIEC has already been determined. Therefore, the length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, the oligonucleotides SEQ ID NO: 5 and SEQ ID NO: 6 of the present invention are used in combination, a nucleotide fragment of 293 bases (or a nucleotide duplex of 293 base pairs) should be amplified. When this estimation accords with the length of the amplified nucleotide sequence, it is judged that the combination of primers accurately amplifies the target region in the *inv*E gene, and that the bacterial strain in the specimen has the *inv*E gene. The results obtained from the agarose gel electrophoresis with 341 test strains are given in Tables 5-1 to 5-7. PCR using the primers of the present invention amplifies only the DNA of the strains which give the *inv*E positive results in the colony hybridization test, showing no amplification of DNAs of *inv*E negative strains. This indicates that PCR using the primers of the present invention is capable of accurately amplifying the *inv*E gene and that *Shigella* species and EIEC both having the *inv*E gene can be detected with high accuracy by using the oligonucleotides of the present invention.

Table 5 — I

No	Strains	CH test*	Primer 5+6 **	No	Strains	CH test*	Primer 5+6 **
001	S. dysenteriae TUMD 1	—	—	026	S. dysenteriae AA-22192	—	—
002	S. dysenteriae TUMD 2	—	—	027	S. dysenteriae AA-22555	+	+
003	S. dysenteriae TUMD 3	—	—	028	S. dysenteriae AA-21933	+	+
004	S. dysenteriae TUMD 4	+	+	029	S. dysenteriae AA-22496	+	+
005	S. dysenteriae TUMD 5	—	—	030	S. dysenteriae AA-22224	—	—
006	S. dysenteriae TUMD 6	—	—	031	S. dysenteriae AA-22542	+	+
007	S. dysenteriae MARABIA	—	—	032	S. dysenteriae AA-22616	—	—
008	S. dysenteriae AQ-7003	—	—	033	S. dysenteriae AA-22239	+	+
009	S. dysenteriae AQ-7004	+	+	034	S. dysenteriae AA-22538	+	+
010	S. dysenteriae AQ-7018	—	—	035	S. dysenteriae ATCC9361	+	+
011	S. dysenteriae AQ-7029	—	—	036	S. dysenteriae ATCC9753	+	+
012	S. dysenteriae AQ-7030	+	+	037	S. dysenteriae ATCC11456a	+	+
013	S. dysenteriae AQ-7061	—	—	038	S. dysenteriae ATCC13313	+	+
014	S. dysenteriae AQ-7125	+	+	039	S. dysenteriae ATCC23351	+	+
015	S. dysenteriae AQ-7131	—	—	040	S. dysenteriae ATCC29027	+	+
016	S. dysenteriae AQ-7151	—	—	041	S. dysenteriae ATCC29028	+	+
017	S. dysenteriae AQ-7164	+	+	042	S. flexneri TUMD 7	—	—
018	S. dysenteriae AQ-7166	+	+	043	S. flexneri TUMD 8	—	—
019	S. dysenteriae AQ-7234	+	+	044	S. flexneri TUMD 9	+	+
020	S. dysenteriae AQ-7302	—	—	045	S. flexneri TUMD10	—	—
021	S. dysenteriae AQ-7350	+	+	046	S. flexneri TUMD11	—	—
022	S. dysenteriae AQ-7370	+	+	047	S. flexneri TUMD12	+	+
023	S. dysenteriae AQ-7403	+	+	048	S. flexneri TUMD13	+	+
024	S. dysenteriae AA-22021	+	+	049	S. flexneri TUMD14	—	—
025	S. dysenteriae AA-22184	—	—	050	S. flexneri TUMD15	+	+

Table 5 — 2

No	Strains	CH test*	Primer 5+6 **	No	Strains	CH test*	Primer 5+6 **
051	S. flexneri TUMD16	—	—	076	S. flexneri TUMD42	+	+
052	S. flexneri TUMD17	—	—	077	S. flexneri TUMD43	+	+
053	S. flexneri TUMD18	—	—	078	S. flexneri TUMD44	+	+
054	S. flexneri TUMD19	+	+	079	S. flexneri TUMD45	+	+
055	S. flexneri TUMD20	—	—	080	S. flexneri TUMD46	—	—
056	S. flexneri TUMD21	—	—	081	S. flexneri TUMD47	+	+
057	S. flexneri TUMD22	—	—	082	S. flexneri TUMD48	—	—
058	S. flexneri TUMD23	—	—	083	S. flexneri TUMD49	+	+
059	S. flexneri TUMD24	+	+	084	S. flexneri TUMD50	—	—
060	S. flexneri TUMD25	—	—	085	S. flexneri TUMD51	—	—
061	S. flexneri TUMD26	—	—	086	S. flexneri TUMD52	+	+
062	S. flexneri TUMD27	—	—	087	S. flexneri TUMD53	—	—
063	S. flexneri TUMD28	—	—	088	S. flexneri TUMD54	—	—
064	S. flexneri TUMD29	—	—	089	S. flexneri TUMD55	—	—
065	S. flexneri TUMD30	+	+	090	S. flexneri TUMD56	—	—
066	S. flexneri TUMD31	—	—	091	S. flexneri TUMD57	—	—
067	S. flexneri TUMD32	—	—	092	S. flexneri TUMD58	—	—
068	S. flexneri TUMD33	—	—	093	S. flexneri TUMD59	—	—
069	S. flexneri TUMD34	—	—	094	S. flexneri TUMD60	—	—
070	S. flexneri TUMD35	+	+	095	S. flexneri TUMD61	—	—
071	S. flexneri TUMD36	+	+	096	S. flexneri Maramba 89-77	—	—
072	S. flexneri TUMD38	—	—	097	S. flexneri Maramba 89-95	+	+
073	S. flexneri TUMD39	+	+	098	S. flexneriMaramba89-109a	—	—
074	S. flexneri TUMD40	—	—	099	S. flexneri Maramba89-119	—	—
075	S. flexneri TUMD41	+	+	100	S. flexneri Maramba89-155	—	—

Table 5 – 3

No	Strains	CH test*	Primer 5+6 **	No	Strains	CH test*	Primer 5+6 **
101	S. flexneri Maramba89-164	–	–	126	S. flexneri AQ-7385	+	+
102	S. flexneri Maramba89-150	+	+	127	S. flexneri AQ-7386	–	–
103	S. flexneri AA-22175	–	–	128	S. flexneri AQ-7390	–	–
104	S. flexneri AA-22371	–	–	129	S. flexneri AQ-7391	+	+
105	S. flexneri AA-22266	+	+	130	S. flexneri AQ-7393	–	–
106	S. flexneri AA-22636	+	+	131	S. flexneri AQ-7394	–	–
107	S. flexneri AA-22187	–	–	132	S. flexneri AQ-7398	–	–
108	S. flexneri AA-22170	+	+	133	S. flexneri AQ-7399	–	–
109	S. flexneri AA-22367	+	+	134	S. flexneri AQ-7400	–	–
110	S. flexneri AA-22316	–	–	135	S. flexneri AQ-7402	–	–
111	S. flexneri AA-22265	+	+	136	S. flexneri AQ-7407	–	–
112	S. flexneri AA-22296	–	–	137	S. flexneri AQ-7408	–	–
113	S. flexneri AA-22312	+	+	138	S. flexneri AQ-7411	+	+
114	S. flexneri AA-22246	–	–	139	S. flexneri AQ-7416	+	+
115	S. flexneri AA-21981	–	–	140	S. flexneri AQ-7417	+	+
116	S. flexneri AA-22097	–	–	141	S. flexneri AQ-7418	+	+
117	S. flexneri AQ-7347	+	+	142	S. flexneri AQ-7423	+	+
118	S. flexneri AQ-7348	+	+	143	S. flexneri AQ-7424	–	–
119	S. flexneri AQ-7351	–	–	144	S. flexneri AQ-7426	+	+
120	S. flexneri AQ-7360	+	+	145	S. flexneri AQ-7427	+	+
121	S. flexneri AQ-7367	+	+	146	S. flexneri Manila 89-164	–	–
122	S. flexneri AQ-7372	+	+	147	S. flexneri Manila 89-177	–	–
123	S. flexneri AQ-7378	–	–	148	S. flexneri Manila 89-209	–	–
124	S. flexneri AQ-7379	–	–	149	S. flexneri Manila 89-210	+	+
125	S. flexneri AQ-7380	–	–	150	S. flexneri Manila 89-229	+	+

Table 5 – 4

No	Strains	CH test*	Primer 5+6 **	No	Strains	CH test*	Primer 5+6 **
151	S. flexneri Manila 89-230	–	–	176	S. boydii TUMD64	+	+
152	S. flexneri Manila 89-231	+	+	177	S. boydii TUMD65	–	–
153	S. flexneri Manila 89-232	+	+	178	S. boydii TUMD66	+	+
154	S. flexneri Manila 89-233	–	–	179	S. boydii TUMD67	–	–
155	S. flexneri Manila 89-273	+	+	180	S. boydii TUMD68	–	–
156	S. flexneri Manila 89-328	+	+	181	S. boydii AQ-7019	–	–
157	S. flexneri Manila 89-333	+	+	182	S. boydii AQ-7020	–	–
158	S. flexneri Manila 89-365	+	+	183	S. boydii AQ-7026	–	–
159	S. flexneri Manila 89-274	–	–	184	S. boydii AQ-7032	+	+
160	S. flexneri Manila 89-436	–	–	185	S. boydii AQ-7039	+	+
161	S. flexneri Manila 89-438	+	+	186	S. boydii AQ-7042	+	+
162	S. flexneri Manila 89-443	+	+	187	S. boydii AQ-7062	+	+
163	S. flexneri Manila 89-444	+	+	188	S. boydii AQ-7076	–	–
164	S. flexneri Manila 89-450	+	+	189	S. boydii AQ-7098	+	+
165	S. flexneri Manila 89-480	+	+	190	S. boydii AQ-7157	+	+
166	S. flexneri Manila 89-483	–	–	191	S. boydii AQ-7193	–	–
167	S. flexneri Manila 89-486	–	–	192	S. boydii AQ-7206	–	–
168	S. flexneri Manila 89-498	–	–	193	S. boydii AQ-7213	+	+
169	S. flexneri Manila 89-499	–	–	194	S. boydii AQ-7218	+	+
170	S. flexneri Manila 89-503	–	–	195	S. boydii AQ-7238	+	+
171	S. flexneri Manila 89-509	–	–	196	S. boydii AQ-7267	–	–
172	S. flexneri Manila 89-532	–	–	197	S. boydii AQ-7268	+	+
173	S. flexneri Manila 89-539	–	–	198	S. boydii AQ-7307	+	+
174	S. boydii TUMD62	–	–	199	S. boydii AQ-7313	–	–
175	S. boydii TUMD63	+	+	200	S. boydii AQ-7314	–	–

Table 5 — 5

No	Strains		CH test*	Primer 5+6 **	No	Strains		CH test*	Primer 5+6 **
201	S. boydii	AQ-7324	−	−	226	S. sonnei	AQ-7382	−	−
202	S. boydii	AQ-7349	+	+	227	S. sonnei	AQ-7383	−	−
203	S. boydii	AQ-7354	−	−	228	S. sonnei	AQ-7384	−	−
204	S. boydii	AQ-7356	−	−	229	S. sonnei	AQ-7387	−	−
205	S. boydii	AQ-7357	+	+	230	S. sonnei	AQ-7388	+	+
206	S. boydii	AQ-7368	+	+	231	S. sonnei	AQ-7389	+	+
207	S. boydii	AQ-7373	+	+	232	S. sonnei	AQ-7392	+	+
208	S. boydii	AQ-7376	−	−	233	S. sonnei	AQ-7395	−	−
209	S. boydii	AQ-7405	+	+	234	S. sonnei	AQ-7396	−	−
210	S. boydii	AA-22562	+	+	235	S. sonnei	AQ-7397	+	+
211	S. boydii	AA-22241	−	−	236	S. sonnei	AQ-7401	+	+
212	S. boydii	AA-22610	−	−	237	S. sonnei	AQ-7406	+	+
213	S. boydii	AA-20255	+	+	238	S. sonnei	AQ-7409	−	−
214	S. boydii	AA-20211	+	+	239	S. sonnei	AQ-7410	−	−
215	S. boydii	AA-21713	−	−	240	S. sonnei	AQ-7412	−	−
216	S. boydii	AA-17405	−	−	241	S. sonnei	AQ-7413	+	+
217	S. boydii	AA-22804	−	−	242	S. sonnei	AQ-7414	−	−
218	S. boydii	AQ-7297	+	+	243	S. sonnei	AQ-7415	−	−
219	S. sonnei	AQ-7366	−	−	244	S. sonnei	AQ-7419	+	+
220	S. sonnei	AQ-7369	+	+	245	S. sonnei	AQ-7420	−	−
221	S. sonnei	AQ-7371	−	−	246	S. sonnei	AQ-7421	+	+
222	S. sonnei	AQ-7374	−	−	247	S. sonnei	AQ-7422	+	+
223	S. sonnei	AQ-7375	+	+	248	S. sonnei	AQ-7425	−	−
224	S. sonnei	AQ-7377	+	+	249	S. sonnei	AA-22634	+	+
225	S. sonnei	AQ-7381	−	−	250	S. sonnei	AA-22677	+	+

Table 5 – 6

No	Strains		CH test*	Primer 5+6 **	No	Strains		CH test*	Primer 5+6 **
251	S. sonnei	AA-18306	–	–	276	S. sonnei	TUMD91	–	–
252	S. sonnei	AA-22067	–	–	277	S. sonnei	TUMD92	+	+
253	S. sonnei	AA-22870	–	–	278	S. sonnei	TUMD93	+	+
254	S. sonnei	TUMD69	+	+	279	S. sonnei	TUMD94	+	+
255	S. sonnei	TUMD70	–	–	280	S. sonnei	TUMD95	–	–
256	S. sonnei	TUMD71	–	–	281	S. sonnei	TUMD96	–	–
257	S. sonnei	TUMD72	–	–	282	S. sonnei	TUMD97	–	–
258	S. sonnei	TUMD73	+	+	283	S. sonnei	TUMD98	–	–
259	S. sonnei	TUMD74	–	–	284	S. sonnei	TUMD99	–	–
260	S. sonnei	TUMD75	+	+	285	S. sonnei	TUMD100	–	–
261	S. sonnei	TUMD76	–	–	286	S. sonnei	TUMD101	–	–
262	S. sonnei	TUMD77	–	–	287	S. sonnei	TUMD102	+	+
263	S. sonnei	TUMD78	+	+	288	S. sonnei	TUMD103	+	+
264	S. sonnei	TUMD79	–	–	289	S. sonnei	TUMD104	+	+
265	S. sonnei	TUMD80	–	–	290	S. sonnei	TUMD105	+	+
266	S. sonnei	TUMD81	+	+	291	S. sonnei	TUMD106	–	–
267	S. sonnei	TUMD82	–	–	292	S. sonnei	TUMD107	+	+
268	S. sonnei	TUMD83	–	–	293	S. sonnei	TUMD108	–	–
269	S. sonnei	TUMD84	–	–	294	S. sonnei	TUMD109	+	+
270	S. sonnei	TUMD85	–	–	295	S. sonnei	TUMD110	–	–
271	S. sonnei	TUMD86	–	–	296	S. sonnei	TUMD111	+	+
272	S. sonnei	TUMD87	–	–	297	S. sonnei	TUMD112	–	–
273	S. sonnei	TUMD88	–	–	298	S. sonnei	TUMD113	+	+
274	S. sonnei	TUMD89	–	–	299	S. sonnei	TUMD114	+	+
275	S. sonnei	TUMD90	+	+	200	S. sonnei	TUMD115	–	–

Table 5 – 7

No	Strains	CH test*	Primer 5+6 **	No	Strains	CH test*	Primer 5+6 **
301	S. sonnei TUMD116	–	–	326	E. coli AQ8011	+	+
302	S. sonnei TUMD117	–	–	327	E. coli AQ8012	+	+
303	S. sonnei TUMD118	+	+	328	E. coli AQ8013	–	–
304	S. sonnei TUMD119	+	+	329	E. coli AQ8016	+	+
305	S. sonnei TUMD120	+	+	330	E. coli AQ8019	+	+
306	S. sonnei TUMD121	–	–	331	E. coli AQ8022	+	+
307	S. sonnei TUMD122	–	–	332	E. coli AQ8024	–	–
308	S. sonnei TUMD123	+	+	333	E. coli AQ8025	+	+
309	S. sonnei TUMD124	+	+	334	E. coli AQ8027	–	–
310	S. sonnei TUMD125	–	–	335	E. coli AQ8028	+	+
311	S. sonnei TUMD126	+	+	336	E. coli AQ8029	+	+
312	S. sonnei TUMD127	–	–	337	E. coli AQ8031	+	+
313	S. sonnei TUMD128	–	–	338	E. coli AQ8033	–	–
314	S. sonnei Maramba 89-154	+	+	339	E. coli AQ8036	–	–
315	S. sonnei Maramba 89-161	–	–	340	E. coli AQ8044	+	+
316	S. sonnei Manila 89-342	–	–	341	E. coli PE660	+	+
317	S. sonnei Manila 89-445	+	+				
318	E. coli DMR 6	+	+				
319	E. coli DMR78	+	+				
320	E. coli DMR79	–	–				
321	E. coli AQ8001	–	–				
322	E. coli AQ8003	+	+				
323	E. coli AQ8004	–	–				
324	E. coli AQ8008	+	+				
325	E. coli AQ8010	+	+				

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to the *inv*E gene, DNAs of clinically important pathogenic bacteria other than *Shigella* species and EIEC are examined with the

primers of the present invention. The same procedure as used in Experiment 1 is followed, except for the procedure of preparation of specimens.

Preparation of specimens

Each strain listed in Table 6 is treated in the same manner as in Experiment 2 of Example 1.

Results

Table 6 shows the results of the test using the combination of primers of the present invention. This combination of primers does not amplify any DNAs of pathogenic bacteria other than *Shigella* species and EIEC. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with DNAs of the bacteria having the *inv*E gene.

Table 6

No	Strains		PCR	No	Strains		PCR
01	Bacillus cereus	ATCC14579	-	21	Klebsiella pneumoniae	JCM1662	-
02	Bacillus Subtilis	JCM1465	-	22	Proteus vulgaris	JCM1668	-
03	Staphylococcus aureus	JCM2413	-	23	Citrobacter freundii	ATCC33128	-
04	Staphylococcus epidermidis	JCM2414	-	24	Streptococcus pyogenes	ATCC12344	-
05	Salmonella typhimurium	IFO12529	-	25	Streptococcus pneumoniae	ATCC33400	-
06	Salmonella enteritidis	IFO3163	-	26	Elaemophilis influenzae	ATCC33391	-
07	Clostridium perfringens	ATCC12917	-	27	Proteus mirabilis	ATCC29906	-
08	Vibrio cholerae	ATCC25872	-	28	Neisseria meningitidis	ATCC13077	-
09	Vibrio cholerae type Ogawa	ATCC9458	-	29	Neisseria gonorthoeae	ATCC19424	-
10	Vibrio cholerae type Inaba	ATCC9459	-	30	Listeria monocytogenes	ATCC15313	-
11	Vibrio fluvialis	JCM3752	-	31	Lactobacillus acidophilus	JCM1132	-
12	Campylobacter jejuni	JCM2013	-	32	Bifidobacterium adolescentis	JCM1275	-
13	Campylobacter coli	JCM2529	-	33	Fusobacterium nucleatum	ATCC25586	-
14	Escherichia coli	JCM1649	-	34	Propionibacterium acnes	ATCC6919	-
15	Yersinia enterocolitica	ATCC9610	-	35	Veillonella atypica	ATCC17744	-
16	Corynebacterium diphtheriae	JCM13	-	36	Pseudomonas aeruginosa	IFO12689	-
17	Peptostreptococcus anaerobius	ATCC273	-	37	Human placental	DNA	-
18	Bacteroides flagilis	ATCC23745	-				
19	Bacteroides vulgatus	JCM5826	-				
20	Enterococcus faecalis	JCM5803	-				

Example 4: Detection of *Salmonella* species having the *ara*C gene

[Experiment 1]

Preparation of specimens

As listed in Tables 7-1 to 7-6, the 133 various *Salmonella* species isolated from the patients and food samples are used. The details are as follows: 67 strains of *Salmonella typhimurium*, 1 of *Salmonella havana*, 2 of *Salmonella oranienburg*, 3 of *Salmonella london*, 3 of *Salmonella senftenberg*, 4 of

Salmonella blockley, 3 of *Salmonella agona*, 4 of *Salmonella infantis*, 14 of *Salmonella litchfield*, 6 of *Salmonella enteritidis*, 13 of *Salmonella thompson*, 6 of *Samonella paratyphi* B, 2 of *Salmonella montevideo*, 1 of *Salmonella gallinarum*, 1 of *Salmonella choleraesuis*, 1 of *Salmonella derby*, 1 of *Salmonella give* and 1 of *Salmonella heidelberg*. Each strain is inoculated to an appropriate medium, and subjected to overnight culture at 37 °C under aerobic conditions. Each culture broth is diluted with TE buffer, and heated at 95 °C for 10 minutes, followed by centrifugation. The supernatants are used as specimens.

Synthesis of primers

As primers for amplifying the *ara*C gene of *Salmonella typhimurium*, the above-described oligonucleotides SEQ ID NO:7 to SEQ ID NO:11 are selected based upon the known base sequence of the *ara*C gene [Horwitz, A. H., et al., Gene 14, 309-319(1981); Clarke, P., et al., Gene 18, 157-163(1982); Lee, J. -H., et al., Gene 46, 113-121 (1986)], and chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that any one of the following oligonucleotide combinations is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:7 + Oligonucleotide SEQ ID NO:8;
Oligonucleotide SEQ ID NO:9 + Oligonucleotide SEQ ID NO:10; and
Oligonucleotide SEQ ID NO:11 + Oligonucleotide SEQ ID NO:8.

Detection

Agarose gel electrophoresis

The same procedure as in Example 1 is followed.

Results

The base sequence of the *ara*C gene of *Salmonella typhimurium* has already been determined. This base sequence is thought to be common to all *Salmonella* species. The length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when oligonucleotides SEQ ID NO: 7 and SEQ ID NO: 8 of the present invention are used in combination, a nucleotide fragment of 361 bases (or a nucleotide duplex of 361 base pairs) is amplified. Similarly, the combination of SEQ ID NO: 9 and SEQ ID NO:10, and that of SEQ ID NO:11 and SEQ ID NO:8 amplify nucleotide fragment of 493 bases and that of 334 bases, respectively. When these estimations accord with the length of the amplified nucleotide fragments, it is judged that PCR using the combination of primers accurately amplifies the target region in the *ara*C gene, and that the bacterial strain in the specimen has the *ara*C gene. Tables 7-1 to 7-6 shows the results of the detection of the *ara*C gene in *Salmonella* species. As obvious from Tables 7-1 to 7-6, the *ara*C gene of *Salmonella* species are detected with high accuracy by using the oligonucleotide primers of the present invention.

Table 7 — 1

No	Strains	Combination of primers*		
		7 + 8**	9 +10**	11+ 8**
001	Salmonella typhimurium 56- 1	+	+	+
002	Salmonella typhimurium 56- 2	+	+	+
003	Salmonella typhimurium 56- 3	+	+	+
004	Salmonella typhimurium 56- 4	+	+	+
005	Salmonella typhimurium 56- 5	+	+	+
006	Salmonella typhimurium 56- 6	+	+	+
007	Salmonella typhimurium 56- 7	+	+	+
008	Salmonella typhimurium 56-11	+	+	+
009	Salmonella typhimurium 56-12	+	+	+
010	Salmonella typhimurium 56-13	+	+	+
011	Salmonella typhimurium 56-17	+	+	+
012	Salmonella typhimurium 56-18	+	+	+
013	Salmonella typhimurium 56-19	+	+	+
014	Salmonella typhimurium 56-20	+	+	+
015	Salmonella typhimurium 56-21	+	+	+
016	Salmonella typhimurium 56-22	+	+	+
017	Salmonella typhimurium 56-23	+	+	+
018	Salmonella typhimurium 56-25	+	+	+
019	Salmonella typhimurium 56-26	+	+	+
020	Salmonella typhimurium 56-27	+	+	+
021	Salmonella typhimurium 56-30	+	+	+
022	Salmonella typhimurium 56-31	+	+	+
023	Salmonella typhimurium 56-32	+	+	+
024	Salmonella typhimurium 57- 3	+	+	+
025	Salmonella typhimurium 57- 4	+	+	+

Table 7 – 2

No	Strains	Combination of primers*		
		7 + 8 **	9 +10**	11 + 8 **
026	Salmonella typhimurium 57- 5	+	+	+
027	Salmonella typhimurium 57- 6	+	+	+
028	Salmonella typhimurium 57- 7	+	+	+
029	Salmonella typhimurium 57- 9	+	+	+
030	Salmonella typhimurium 57-10	+	+	+
031	Salmonella typhimurium 57-11	+	+	+
032	Salmonella typhimurium 57-19	+	+	+
033	Salmonella typhimurium 57-20	+	+	+
034	Salmonella typhimurium 59-26	+	+	+
035	Salmonella typhimurium 59-27	+	+	+
036	Salmonella typhimurium 59-28	+	+	+
037	Salmonella typhimurium 59-54	+	+	+
038	Salmonella typhimurium 59-55	+	+	+
039	Salmonella typhimurium 59-56	+	+	+
040	Salmonella typhimurium 59-57	+	+	+
041	Salmonella typhimurium 59-58	+	+	+
042	Salmonella typhimurium 60- 5	+	+	+
043	Salmonella typhimurium 60- 6	+	+	+
044	Salmonella typhimurium 60- 7	+	+	+
045	Salmonella typhimurium 60-13	+	+	+
046	Salmonella typhimurium 61- 1	+	+	+
047	Salmonella typhimurium 61-16	+	+	+
048	Salmonella typhimurium 62- 1	+	+	+
049	Salmonella typhimurium 62- 2	+	+	+
050	Salmonella typhimurium 62- 3	+	+	+

Table 7 − 3

No	Strains	Combination of primers*		
		7 + 8 **	9 +10**	11 + 8 **
051	Salmonella typhimurium 62- 4	+	+	+
052	Salmonella typhimurium 62- 5	+	+	+
053	Salmonella typhimurium 62- 6	+	+	+
054	Salmonella typhimurium 63- 6	+	+	+
055	Salmonella typhimurium 63- 7	+	+	+
056	Salmonella typhimurium 63- 8	+	+	+
057	Salmonella typhimurium 63- 9	+	+	+
058	Salmonella typhimurium 89- 1	+	+	+
059	Salmonella typhimurium 89- 2	+	+	+
060	Salmonella typhimurium IFO12529	+	+	+
061	Salmonella typhimurium IFO13245	+	+	+
062	Salmonella typhimurium IFO14193	+	+	+
063	Salmonella typhimurium IFO14194	+	+	+
064	Salmonella typhimurium IFO14209	+	+	+
065	Salmonella typhimurium IFO14210	+	+	+
066	Salmonella typhimurium IFO14211	+	+	+
067	Salmonella typhimurium IFO14212	+	+	+
068	Salmonella litchfield 56- 8	+	+	+
069	Salmonella litchfield 59-25	+	+	+
070	Salmonella litchfield 53-22	+	+	+
071	Salmonella litchfield 53-23	+	+	+
072	Salmonella litchfield 53-24	+	+	+
073	Salmonella litchfield 54- 5	+	+	+
074	Salmonella litchfield 54- 6	+	+	+
075	Salmonella litchfield 55- 3	+	+	+

Table 7 − 4

No	Strains	Combination of primers*		
		7 + 8 **	9 +10**	11 + 8 **
076	Salmonella litchfield 55- 4	+	+	+
077	Salmonella litchfield 55- 6	+	+	+
078	Salmonella litchfield 55- 7	+	+	+
079	Salmonella litchfield 55- 8	+	+	+
080	Salmonella litchfield 55-12	+	+	+
081	Salmonella litchfield 55-13	+	+	+
082	Salmonella thompson 61- 2	+	+	+
083	Salmonella thompson 61- 3	+	+	+
084	Salmonella thompson 61- 4	+	+	+
085	Salmonella thompson 61-17	+	+	+
086	Salmonella thompson 61-18	+	+	+
087	Salmonella thompson 52- 3	+	+	+
088	Salmonella thompson 52- 4	+	+	+
089	Salmonella thompson 53- 5	+	+	+
090	Salmonella thompson 53- 6	+	+	+
091	Salmonella thompson 53- 7	+	+	+
092	Salmonella thompson 53-20	+	+	+
093	Salmonella thompson 53-21	+	+	+
094	Salmonella thompson NIAH1230	+	+	+
095	Salmonella enteritidis 59-36	+	+	+
096	Salmonella enteritidis 59-37	+	+	+
097	Salmonella enteritidis 59-38	+	+	+
098	Salmonella enteritidis 53- 1	+	+	+
099	Salmonella enteritidis 53- 2	+	+	+
100	Salmonella enteritidis IFO3313	+	+	+

Table 7 – 5

No	Strains		Combination of primers*		
			7 + 8 **	9 +10**	11+ 8 **
101	Salmonella paratyphi B	61-19	+	+	+
102	Salmonella paratyphi B	61-20	+	+	+
103	Salmonella paratyphi B	61-21	+	+	+
104	Salmonella paratyphi B	63- 1	+	+	+
105	Salmonella paratyphi B	63- 2	+	+	+
106	Salmonella paratyphi B	63- 3	+	+	+
107	Salmonella blockley	58-55	+	+	+
108	Salmonella blockley	58-56	+	+	+
109	Salmonella blockley	58-57	+	+	+
110	Salmonella blockley	NIAH1197	+	+	+
111	Salmonella infantis	59-20	+	+	+
112	Salmonella infantis	59-21	+	+	+
113	Salmonella infantis	59-22	+	+	+
114	Salmonella infantis	NIAH1218	+	+	+
115	Salmonella agona	59- 1	+	+	+
116	Salmonella agona	59- 2	+	+	+
117	Salmonella agona	59- 3	+	+	+
118	Salmonella london	58- 7	+	+	+
119	Salmonella london	58- 8	+	+	+
120	Salmonella london	58- 9	+	+	+
121	Salmonella senftenberg	58-27	+	+	+
122	Salmonella senftenberg	58-28	+	+	+
123	Salmonella senftenberg	58-29	+	+	+
124	Salmonella oranienburg	57- 1	+	+	+
125	Salmonella oranienburg	57- 2	+	+	+

Table 7 – 6

No	Strains	Combination of primers*		
		7 + 8**	9 +10**	11+ 8 **
126	Salmonella montevideo 54- 4	+	+	+
127	Salmonella montevideo NIAH1221	+	+	+
128	Salmonella gallinarum IFO3163	+	+	+
129	Salmonella choleraesuis NIAH1198	+	+	+
130	Salmonella derby NIAH1199	+	+	+
131	Salmonella give NIAH1214	+	+	+
132	Salmonella havana 56-44	+	+	+
133	Salmonella heiderberg NIAH1216	+	+	+

Note) * + : DNA of estimated length is amplified.
N : DNA of not-estimated length is amplified.
– : DNA is not amplified.
** Numerals refer to SEQ ID NOs.

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to the *ara*C gene of *Salmonella* species, DNAs of clinically important diarrheal bacteria other than *Salmonella* species and other pathogenic bacteria are examined with the primers of the present invention. In particular, differentiation between *Salmonella* species and *Citrobacter* species, which has been difficult by conventional methods, is carefully examined.

The same procedure as used in Experiment 1 is followed, except for the procedure of preparation of specimens.

Preparation of specimens

Clostridium perfringens, *Campylobacter jejuni*, *Campylobacter coli*, *Bacteroides fragilis*, *Bacteroides vulgatus*, *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* are cultured at 37 °C under anaeorbic conditions, while *Neisseria gonorrhoeae* and *Neisseria meningitidis* are cultured in the presence of 3-10% CO_2.

Human placenta DNA, at a concentration of 1 μg/ml, is subjected to PCR in the same manner as above.

Results

Tables 8-1 to 8-3 shows the results of the test using the combinations of the primers of the present invention. These combinations of primers do not amplify any DNAs of bacterial strains other than *Salmonella* species or DNAs of human placenta. It is of particular importance that the combinations of the primers of the present invention do not amplify any DNAs of *Citrobacter* species which are closely akin to and hardly differentiated from *Salmonella* species. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with the DNAs of *Salmonella* species, with high reliability.

Table 8 − 1

No	Strains		Combination of primers*		
			7 + 8**	9 +10**	11+ 8**
01	Bacillus cereus	ATCC14579	−	−	−
02	Bacillus subtilis	JCM1465	−	−	−
03	Staphylococcus aureus	JCM2413	−	−	−
04	Staphylococcus epidermidis	JCM2414	−	−	−
05	Clostridium perfringens	ATCC12917	−	−	−
06	Vibrio cholerae	ATCC25872	−	−	−
07	Vibrio cholerae type Ogawa	ATCC9458	−	−	−
08	Vibrio cholerae type Inaba	ATCC9459	−	−	−
09	Vibrio cholerae	61H-151	−	−	−
10	Vibrio parahaemolyticus	WP-1	−	−	−
11	Vibrio fluvialis	JCM3752	−	−	−
12	Campylobacter jejuni	JCM2013	−	−	−
13	Campylobacter coli	JCM2529	−	−	−
14	Escherichia coli	JCM1649	−	−	−
15	Escherichia coli	H10407	−	−	−
16	Escherichia coli	WHO 3	−	−	−
17	Escherichia coli	WHO 47	−	−	−
18	Escherichia coli	T- 1	−	−	−
19	Escherichia coli	T-40	−	−	−
20	Yersinia enterocolitica	ATCC9610	−	−	−
21	Shigella dysenteriae	ATCC9361	−	−	−
22	Shigella boydii	ATCC9210	−	−	−
23	Shigella flexneri	ATCC11836	−	−	−
24	Shigella sonnei	ATCC9290	−	−	−
25	Bacteroides flagilis	ATCC23745	−	−	−

Table 8 − 2

No	Strains		Combination of primers*		
			7 + 8**	9 + 10**	11 + 8**
26	Bacteroides vulgatus	JCM5826	−	−	−
27	Proteus vulgaris	JCM1668	−	−	−
28	Proteus mirabilis	ATCC29906	−	−	−
29	Streptococcus pyogenes	ATCC12344	−	−	−
30	Streptococcus pneumoniae	ATCC33400	−	−	−
31	Heamophilis influenzae	ATCC33391	−	−	−
32	Klebsiella pneumoniae	JCM1662	−	−	−
33	Neisseria gonorrbeae	ATCC19424	−	−	−
34	Neisseria meningitidis	ATCC13077	−	−	−
35	Listeria monocytogenes	ATCC15313	−	−	−
36	Lactobacillus acidophilus	JCM1132	−	−	−
37	Bifidobacterium adolescentis	JCM1275	−	−	−
38	Fusobacterium nucleatum	ATCC25586	−	−	−
39	Propionibacterium acnes	ATCC6919	−	−	−
40	Veillonella atypica	ATCC17744	−	−	−
41	Pseudomonas aeruginosa	IFO12689	−	−	−
42	Corynebacterium diphtheriae	JCM1310	−	−	−
43	Peptostreptococcus anaerobius	ATCC27337	−	−	−
44	Citrobacter freundii	ATCC6750	−	−	−
45	Citrobacter freundii	ATCC6879	−	−	−
46	Citrobacter freundii	ATCC8090	−	−	−
47	Citrobacter freundii	ATCC8454	−	−	−
48	Citrobacter freundii	ATCC10053	−	−	−
49	Citrobacter freundii	ATCC10625	−	−	−
50	Citrobacter freundii	ATCC10787	−	−	−

Table 8 − 3

No	Strains		Combination of primers*		
			7 + 8**	9 +10**	11+ 8**
51	Citrobacter freundii	ATCC11102	−	−	−
52	Citrobacter freundii	ATCC11811	−	−	−
53	Citrobacter freundii	ATCC29063	−	−	−
54	Citrobacter freundii	ATCC29219	−	−	−
55	Citrobacter freundii	ATCC29220	−	−	−
56	Citrobacter freundii	ATCC29221	−	−	−
57	Citrobacter freundii	ATCC29222	−	−	−
58	Citrobacter freundii	ATCC29935	−	−	−
59	Citrobacter freundii	ATCC33128	−	−	−
60	Citrobacter amalonaticus	ATCC25405	−	−	−
64	Citrobacter amalonaticus	ATCC25406	−	−	−
64	Citrobacter amalonaticus	ATCC25407	−	−	−
65	Citrobacter diversus	ATCC27156	−	−	−
65	Citrobacter diversus	ATCC29223	−	−	−
65	Citrobacter diversus	ATCC29224	−	−	−
66	Citrobacter diversus	ATCC29225	−	−	−
67	Citrobacter diversus	ATCC29936	−	−	−

Note) * + : DNA of estimated length is amplified.
N : DNA of not-estimated length is amplified.
− : DNA is not amplified.
** Numerals refer to SEQ ID NOs.

Example 5: Detection of EHEC (VTEC) having the VT1 gene

[Experiment 1]

Preparation of specimens

The same procedure as used in Example 1 is followed except that 320 strains of EHEC (VTEC) strains.

Synthesis of primers

As primers for amplifying the VT1 gene of EHEC (VTEC), the above-described oligonucleotides SEQ ID NO:12 and SEQ ID NO:13 are selected based upon the known base sequence of the VT1 gene [Takao T., et al., Microb. Pathog., 5, 357-369(1988)]. These oligonucleotides are chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that the following oligonucleotide combination is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:12 + Oligonucleotide SEQ ID NO:13

Detection

Agarose gel electrophoresis

The same procedure as in Example 1 is followed.

Colony hybridization test

A colony hybridization test is carried out using an oligonucleotide probe specific to the VT1 gene and that specific to the VT2 gene according to the procedure described by Grunstein [Grunstein, M. and Hogness, D., Proc. Natl. Acad. Sci., 72, 3961(1975)].

Results

The base sequence of the VT1 gene of EHEC(VTEC) has already been determined. Therefore, the length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when the oligonucleotides SEQ ID NO:12 and SEQ ID NO:13 of the present invention are used in combination, a nucleotide fragment of 349 bases (or a nucleotide duplex of 349 base pairs) should be amplified. When this estimation accords with the length of the amplified nucleotide fragment, it is judged that PCR using the combination of primers accurately amplifies the target region in the VT1 gene, and that the bacterial strain in the specimen has the VT1 gene. The results obtained from the agarose gel electrophoresis and from the colony hybridization test for 320 test strains are given in Table 9. Table 9 shows that PCR using the primers of the present invention amplifies only DNAs of the strains which give a positive result for the VT1 gene in the colony hybridization test, and that it does not amplify the DNA of the VT1 negative strains. This indicates that PCR using the primers of the present invention is capable of accurately amplifying the VT1 gene and that EHEC(VTEC) having the VT1 gene can be detected with high accuracy by using the oligonucleotides of the present invention.

Table 9

Accuracy of the primers					
		Results of colony hybridization test			
		Positive for VT1 gene	Positive for VT2 gene	Positive for both VT1 and VT2 genes	Negative for both VT1 and VT2 genes
Results of PCR	Positive	39	0	53	0
	Negative	0	185	0	43

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to EHEC (VTEC) having the VT1 gene, the DNAs of clinically important pathogenic bacteria other than EHEC (VTEC) are examined with the primers of the present invention. The same procedure as used in Experiment 1 is followed, except for the procedure of preparation of specimens.

Preparation of specimens

Each strain listed in Table 10 is treated in the same manner as in Experiment 2 of Example 1. Among strains listed in Table 10, the following strains are cultured under anaerobic conditions: *Clostridium perfringens*, *Campylobacter jejuni*, *Bacteroides fragilis*, *Bacteroides vulgatus* and *Lactobacillus acidophilus*.

Results

Table 10 shows the results from the test using the combinations of primers of the present invention. Although the combinations of primers do not amplify DNAs of any other strains than EHEC(VTEC) except for a certain type of *Shigella* species (*Shigella dysenteriae* type I).

It is well known that the differentiation between EHEC (VTEC) and *Shigella dysenteriae* is impossible because *Shigella dysenteriae* has the VT1 gene. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with the DNAs of the bacteria having the VT1 gene.

Table 10. Reactivity with un-targeted gene

No	Strains		Combination of primers			
			12+13*	14+15*	16+18*	17+18*
1	Bacillus cereus	ATCC 14579	−	−	−	−
2	B. subtilis	JCM 1465	−	−	−	−
3	Staphylococcus aureus	JCM 2413	−	−	−	−
4	S. epidermidis	JCM 2414	−	−	−	−
5	Salmonella typhimurium	IFO 12529	−	−	−	−
6	S. enteritidis	IFO 3163	−	−	−	−
7	Clostridium perfringens	ATCC 12917	−	−	−	−
8	Vibrio fluvialis	JCM 3752	−	−	−	−
9	Campylobacter jejuni	JCM 2013	−	−	−	−
10	C. coli	JCM 2529	−	−	−	−
11	Escherichia coli	JCM 1649	−	−	−	−
12	Yersinia enterocolitica	ATCC 9610	−	−	−	−
13	Shigella dysenteriae	ATCC 9361	+	−	+	+
14	S. flexneri	ATCC 29903	−	−	−	−
15	S. sonnei	ATCC 29930	−	−	−	−
16	Bacteroides fragilis	ATCC 23745	−	−	−	−
17	B. vulgatus	JCM 5826	−	−	−	−
18	Enterococcus faecalis	JCM 5803	−	−	−	−
19	Klebsiella pneumoniae	JCM 1662	−	−	−	−
20	Proteus vulgaris	JCM 1668	−	−	−	−
21	Citrobacter freundii	ATCC 33128	−	−	−	−
22	Streptococcus pyogenes	ATCC 12344	−	−	−	−
23	S. pneumoniae	ATCC 33400	−	−	−	−
24	Haemophilus influenzae	ATCC 33391	−	−	−	−
25	Proteus mirabilis	ATCC 29906	−	−	−	−
26	Neisseria gonorrhoeae	ATCC 19424	−	−	−	−
27	N. meningitidis	ATCC 13077	−	−	−	−
28	Listeria monocytogenes	ATCC 15313	−	−	−	−
29	Lactobacillus acidophilus	JCM 1132	−	−	−	−
30	Bifidobacterium adolescentis	JCM 1275	−	−	−	−
31	Fusobacterium nucleatum	ATCC 2558	−	−	−	−
32	Propionibacterium acnes	ATCC 6919	−	−	−	−
33	Veillonella atypica	ATCC 17744	−	−	−	−
34	Pseudomonas aeruginosa	IFO 12689	−	−	−	−
35	Corynebacterium diphtheriae	JCM 1310	−	−	−	−
36	Peptostreptococcus anaerobius	ATCC 27337	−	−	−	−
37	Vibrio cholerae	ATCC 25872	−	−	−	−
38	V. cholerae type Ogawa	ATCC 9458	−	−	−	−
39	V. chalerae type Inaba	ATCC 9459	−	−	−	−

Note) * Numerals refer to SEQ ID NOs.
‡‡ + : reactive
− : nonreactive

Example 6: Detection of EHEC (VTEC) having the VT2 gene

[Experiment 1]

Preparation of specimens

The same procedure as used in Experiment 1 of Example 5 is followed.

Synthesis of primers

As primers for amplifying the VT2 gene of EHEC (VTEC) strains, the above-described oligonucleotides SEQ ID NO:14 and SEQ ID NO:15 are selected based upon the known base sequence of the VT2 gene [Jackson, M.P., et al., FEMS Microbio. Lett., 44, 109-114(1987)]. These oligonucleotides are chemically synthesized by the same method as in Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that the following oligonucleotide combination is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:14 + Oligonucleotide SEQ ID NO:15

Detection

Agarose gel electrophoresis

The same procedure as in Example 1 is followed.

Colony hybridization test

The same procedure as in Experiment 1 of Example 5 is followed.

Results

The base sequence of the VT2 gene of EHEC(VTEC) has already been determined. Therefore, the length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when oligonucleotides SEQ ID NO:14 and SEQ ID NO:15 of the present invention are used in combination, a nucleotide fragment of 404 bases (or a nucleotide duplex of 404 base pairs) should be amplified. When this estimation accords with the length of the amplified nucleotide fragment, it is judged that PCR using the combination of primers accurately amplifies the target region in the VT2 gene, and that the bacterial strain in the specimen has the VT2 gene. The results obtained from the agarose gel electrophoresis with 320 test strains and from the colony hybridization test are given in Table 11. PCR using the primers of the present invention amplifies only DNAs of the strains which give a result positive for the VT2 gene in the colony hybridization test, showing no amplification of the DNA of the VT2 negative strains. This indicates that PCR using the primers of the present invention is capable of accurately amplifying the VT2 gene and that EHEC(VTEC) having the VT2 gene can be detected with high accuracy by using the oligonucleotides of the present invention.

Table 11

Accuracy of the primers					
		Results of colony hybridization test			
		Positive for VT1 gene	Positive for VT2 gene	Positive for both VT1 and VT2 genes	Negative for both VT1 and VT2 genes
Results of PCR	Positive	0	185	53	0
	Negative	39	0	0	43

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to EHEC (VTEC) having the VT2 gene, the DNAs of clinically important pathogenic bacteria other than EHEC (VTEC) are examined with the primers of the present invention. The same procedure as used in Experiment 2 of Example 5 is followed.

Results

Table 10 shows the results of the test using the combinations of primers of the present invention. All the combinations of primers in Table 10 do not amplify the DNAs of pathogenic bacteria other than EHEC (VTEC). It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with the DNAs of the bacteria having the VT2 gene.

Example 7: Detection of EHEC (VTEC) having the VT1 gene, the VT2 gene or a variant gene of the VT2 gene

[Experiment 1]

Preparation of specimens

The same procedure as used in Experiment 1 of Example 1 is followed.

Synthesis of primers

As primers for amplifying the VT1 gene, the VT2 gene or a variant gene of VT2 (VT2vha, VT2vhb or VT2vp1), the above-described oligonucleotides SEQ ID NO:16, SEQ ID NO:17 and SEQ ID NO:18 are selected. These oligonucleotides are chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that any one of the following oligonucleotide combinations is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:16 + Oligonucleotide SEQ ID NO:18; and
Oligonucleotide SEQ ID NO:17 + Oligonucleotide SEQ ID NO:18.

Detection

Agarose gel electrophoresis

The same procedure as in Experiment 1 of Example 1 is followed.

Colony hybridization test

The same procedures as in Experiment 1 of Example 1 are followed.

Results

The base sequences of the VT1 gene, the VT2 gene, the VT2vha gene, the VT2vhb gene and the VT2vp1 gene of EHEC(VTEC) have already been determined. Therefore, the length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when the oligonucleotides SEQ ID NO:16 and SEQ ID NO:18 of the present invention are used in combination, a nucleotide fragment of 495 bases (or a nucleotide duplex of 495 base pairs) should be amplified. When this estimation accords with the length of the amplified nucleotide fragment, it is judged that PCR using the combination of primers accurately amplifies the target region in the VT1 gene, the VT2 gene, the VT2vha gene, the VT2vhb gene or the VT2vp1 gene, and that some bacterial strains in the specimen have any one of these genes. The results obtained from the agarose gel electrophoresis and from the colony hybridization test with 320 test strains are given in Table 12. PCR using the primers of the present invention amplifies only DNA of the strains which give the positive result for the VT1 gene or the VT2 gene, showing no amplification of the DNA of the strains negative for these genes. This indicates that PCR using the primers of the present invention is capable of accurately amplifying the VT1 gene or the VT2 gene (including its variant genes) and that EHEC(VTEC) having the VT1 gene or the VT2 gene or the both can be detected with high accuracy by using the oligonucleotides of the present invention.

Table 12

Accuracy of the primers					
				Results of colony hybridization test	
				Positive for VT1 or VT2 gene	Negative for both VT1 and VT2 genes
Results of PCR	Combination of primers	16 + 18*	Positive	277	0
			Negative	0	43
		17 + 18*	Positive	277	0
			Negative	0	43

Note)
* Numerals refer to SEQ ID NOs.

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to EHEC (VTEC) having the VT1 gene or the VT2 gene, DNAs of clinically important pathogenic bacteria other than EHEC (VTEC) are examined with the primers of the present invention. The same procedure as used in Experiment 2 of Example 5 is followed.

Results

Table 10 shows the results from the test using the combinations of the primers of the present invention. PCR using the combinations of the primers does not amplify DNAs of any other strains than EHEC (VTEC) except for the DNA of a certain type of *Shigella* species (*Shigella dysenteriae* type I).

It is well known that differentiation between EHEC (VTEC) and *Shigella dysenteriae* is impossible only by detecting the VT1 gene because not only EHEC (VTEC) but also *Shigella dysenteriae* has the VT1 gene. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with the DNAs of the bacteria having the VT1 gene or the VT2 gene.

Example 8: Detection of *Staphylococcus aureus* having the TSST-1 gene

[Experiment 1]

Preparation of specimens

A total of 343 strains of *Staphylococcus aureus* are used. These strains are derived from food poisoning cases and the environment, and isolated from sources such as diarrheal stool, vomit and food. Each strain is inoculated to a brain heart infusion medium (manufactured by BBL Co., Ltd.), and subjected to overnight shaking culture at 37 °C under aerobic conditions. Each culture broth is diluted 10 folds with TE buffer, and heated at 95 °C for 5 minutes, followed by centrifugation at 5000 rpm for 1 minute; the supernatants are used as specimens.

Synthesis of primers

As primers for amplifying the TSST-1 gene of *Staphylococcus aureus*, the above-described oligonucleotides SEQ ID NO:19 to SEQ ID NO:22 are selected based upon the known base sequences of the TSST-1 gene of *Staphylococcus aureus* [Blomster-Hautamaa et al., J. Biol. chem., 26, 15783-15786 (1986)], and chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

To 3 μl of the above-described specimen solution, 16.05 μl of sterile distilled water, 3 μl of 10 x reaction buffer, 4.8 μl of dNTP solution, 1.0 μl of primer (1), 1.0 μl of primer (2), and 0.15 μl of a thermostable DNA polymerase are added to prepare 30 μl of a reaction mixture. This reaction mixture is overlaid with 50 μl of mineral oil (produced by SIGMA). The contents of the solutions used and the primers (1) and (2) are as follows:

10 x reaction buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.1% (w/v) gelatin.

dNTP solution: A mixture of dATP, dCTP, dGTP and dTTP, each having a final concentration of 1.25 mM.

Primers: Aqueous solution of the above-described chemically synthesized purified oligonucleotides (concentration, 3.75 OD/ml) is prepared. Any one of the following oligonucleotide combinations is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:20 + Oligonucleotide SEQ ID NO:21
Oligonucleotide SEQ ID NO:19 + Oligonucleotide SEQ ID NO:22 and
Oligonucleotide SEQ ID NO:20 + Oligonucleotide SEQ ID NO:22

Thermostable DNA polymerase: Taq DNA polymerase (5 unit/ml; produced by Perkin Elmer Cetus).

The reaction conditions are as follows:

Thermal denaturation: 94 °C for 1 minute.
Annealing: 55 °C for 1 minute.
Polymerization: 72 °C for 1 minute.

The cycle of thermal denaturation, primer annealing and polymerization (5.7 minutes) is repeated for 35 cycles (entire time, about 3 hours). This procedure is performed using a DNA thermal cycler (produced by Perkin Elmer Cetus) in which the above reaction conditions are programmed.

Detection

Agarose gel electrophoresis

The same procedure as in Example 1 is followed.

Reversed passive latex agglutination (RPLA) test

A commercially available RPLA kit for detection of TSST-1 of *Staphylococcus aureus* (TST-RPLA "SEIKEN" produced by DENKA SEIKEN) is purchased. Specimens are prepared and tested according to the instruction manual attached except that the preparation of specimens is partially modified in order for the test strains to produce sufficient amount of its enterotoxin. That is, the brain heart infusion is changed to the one produced by BBL Co., Ltd.

Results

Table 13 shows the comparison of the results of the PCR method of the present invention with the results of the TST-RPLA which are conventionally used. The data indicates that the detection method of the present invention can detect the TSST-1 gene of *Staphylococcus aureus* with a sensitivity comparable to or higher than the conventional TST-RPLA method. The data in Table 13 show that 17 of the 18 PCR positive strains are also positive by RPLA, and that 325 strains are negative by both PCR and RPLA. That is, except one strain which is positive by PCR and negative by RPLA, the same results are obtained by PCR and by RPLA. The strain, for which the result by PCR and that by RPLA disaccord with each other, is tested by the Southern blot hybridization, and is confirmed to be positive for the TSST-1 gene.

Table 13

Comparison of PCR and TST-RPLA		
	PCR positive	PCR negative
TST-RPLA positive	17	0
TST-RPLA negative	1	325

Figure 1 shows the results of the sensitivity test for the PCR primers of the present invention by electrophoresis. In the figure, numerals 1 to 9 indicate the number of DNA copies used in the PCR reaction: 1 indicates 10^7 copies; 2, 10^6 copies; 3, 10^5 copies; 4, 10^4 copies; 5, 10^3 copies; 6, 10^2 copies; 7, 10 copies; 8, 1 copy; and 9, absence of DNA. From this figure, it is obvious that only 10 copies of DNA can be detected by the method of the present invention.

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to *Staphylococcus aureus* having the TSST-1 gene, DNAs of other clinically important pathogenic bacteria are examined with the primers of the present invention. The same procedure as used in Experiment 1 is followed, except for the method of preparation of specimens.

Preparation of specimens

Each strain listed in Table 14 is treated in the same manner as in Experiment 2 of Example 5.

Results

Table 14 shows the results of the test using the primers of the present invention. PCR using the primers does not amplify any DNAs of other strains including those causative for food poisoning. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with the DNA of *Staphylococcus aureus* having the TSST-1 gene. The similar results are obtained with the other combinations of the primers of the present invention which are not listed in Table 14.

Table 14.

No	Strains		Combination of primers		
			20+21*	19+22*	20+22*
1	Bacillus cereus	ATCC 14579	—	—	—
2	Bacillus subtilis	JCM 1455	—	—	—
3	Staphylococcus aureus	JCM 2413	—	—	—
4	Staphylococcus epidermidis	JCM 2414	—	—	—
5	Salmonella typhimurium	IFO 12529	—	—	—
6	Salmonella enteritidis	IFO 3163	—	—	—
7	Clostridium perfringens	ATCC 12917	—	—	—
8	Vibrio cholerae	ATCC 25872	—	—	—
9	Vibrio cholerae type Ogawa	ATCC 9458	—	—	—
10	Vibrio cholerae type Inaba	ATCC 9459	—	—	—
11	Vibrio fluvialis	JCM 3752	—	—	—
12	Campylobacter jejuni	JCM 2013	—	—	—
13	Campylobacter coli	JCM 2529	—	—	—
14	Eschericia coli	JCM 1548	—	—	—
15	Yersinia enterocolitica	ATCC 5610	—	—	—
16	Shigella dysenteriae	ATCC 3361	—	—	—
17	Shigella flexneri	ATCC 29903	—	—	—
18	Shigella sonnei	ATCC 29930	—	—	—
19	Bacteroides fragilis	ATCC 23745	—	—	—
20	Bacteroides vulgatus	JCM 5826	—	—	—
21	Enterococcus faecalis	JCM 5803	—	—	—
22	Klebsiella pneumoniae	JCM 1662	—	—	—
23	Protaus vulgaris	JCM 1688	—	—	—
24	Citrobacter freundii	ATCC 33128	—	—	—
25	Streptococcus pyogenes	ATCC 12344	—	—	—
26	Streptococcus pneumoniae	ATCC 33400	—	—	—
27	Haemophilus influenzae	ATCC 33391	—	—	—
28	Proteus mirabilis	ATCC 29906	—	—	—
29	Neisseria gonorrhoeae	ATCC 19424	—	—	—
30	Neisseria meningitidis	ATCC 13077	—	—	—
31	Listeria monocytogenes	ATCC 15313	—	—	—
32	Lactobacillus acidophilus	JCM 1132	—	—	—
33	Bifidobacterium adolescentis	JCM 1275	—	—	—
34	Fusobacterium nucleatum	ATCC 25585	—	—	—
35	Propionibacierium acnes	ATCC 5918	—	—	—
36	Veillonella atypica	ATCC 17744	—	—	—
37	Pseudomonas aeruginosa	IFO 12689	—	—	—
38	Corynebacterium diphtheriae	JCM 1310	—	—	—
39	Peptostreptococcus anaerobius	ATCC 27337	—	—	—

Note) * Numerals refer to SEQ ID NOs.

Example 9: Detection of *Vibrio cholerae* having the *ctx* gene

[Experiment 1]

Preparation of specimens

The same procedure as used in Example 1 is followed except that 622 strains of *Vibrio cholerae* are used. These strains are isolated from patients with cholera, marine products (shrimp, snapping turtle), water collected from river, harbor, etc. Serotype, biotype, the numbers of the strains are listed in Table 15.

Table 15. Type and sources of Vibrio cholerae

Serotype		Biotype	Sources			Total
			Patients	Food	Environment water	
O 1	Ogawa	El Tor	148	125	71	344
	Inaba		16	27	26	69
	Ogawa	Asia (classical)	15	0	0	15
	Inaba		26	0	0	26
non O 1		——	168	0	0	168
		Total	373	152	97	622

Synthesis of primers

As primers for amplifying the *ctx* gene of *Vibrio cholerae*, the above-described oligonucleotides SEQ ID NO:23 to SEQ ID NO:26 are selected based upon the known base sequences of the *ctx* gene of *Vibrio cholerae* [Lockman, H. and J.B. Kaper: J. Biol. Chem., 258, 13722-13726 (1983)], and chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that any one of the following oligonucleotide combinations is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:23 + Oligonucleotide SEQ ID NO:25; and
Oligonucleotide SEQ ID NO:24 + Oligonucleotide SEQ ID NO:26.

Detection

Agarose gel electrophoresis

To detect the amplified nucleotide fragments in the reaction solution, agarose gel electrophoresis is carried out by the same procedure as in Example 1.

Colony hybridization test

A colony hybridization test is carried out using an polynucleotide probe specific to the *ctx* gene [Kaper, J.B., J.G. Morris, Jr., and N. Nishibuchi (1988), DNA probes for pathogenic Vibrio species, 65-77. In F.C. Tenover (ed.), DNA probes for infectious diseases. CRC Press, Inc., Boca Raton, Fla.] according to the procedure described by Grunstein [Grunstein, M. and Hogness, D., Proc. Natl. Acad. Sci., 72, 3961(1975)].

Results

The base sequences of the *ctx* gene of *Vibrio cholerae* have already been determined. Therefore, the length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when the oligonucleotides SEQ ID NO: 23 and SEQ ID NO:25 of the present invention are used in combination, a nucleotide fragment of 169 bases (or a nucleotide duplex of 169 base pairs) should be amplified. The combination of SEQ ID NO: 24 and SEQ ID NO:26 should amplify a nucleotide fragment of 307 bases (or a nucleotide duplex of 307 base pairs). When the estimated length of nucleotide accords with the length of the amplified nucleotide fragments, it is judged that PCR using the combination of primers accurately amplifies the target region in the *ctx* gene, and that the bacterial strain in the specimen has the *ctx* gene. The results obtained from the agarose gel electrophoresis and from the colony hybridization test with 662 test strains are given in Table 16. PCR using the primers of the present invention amplifies only DNAs of the strains which give a result positive for the *ctx* gene in the colony hybridization test, showing no amplification of the DNA of the *ctx* gene negative strains. This indicates that PCR using the primers of the present invention is capable of accurately amplifying the *ctx* gene and that *Vibrio cholerae* having the ctx gene can be detected with high accuracy by using the oligonucleotides of the present invention. Table 16 shows the result obtained with oligonucleotides SEQ ID NO: 24 and SEQ ID NO: 26. The combination of SEQ ID NO:23 and SEQ ID NO:25 also gives a similar result.

Table 16

Accuracy of primer combination of SEQ ID NO:24 and SEQ ID NO:26.			
		Results of colony hybridization test	
		ctx gene positive	*ctx* gene negative
Results of PCR	positive	412	0
	negative	0	210

Figure 2 shows that PCR using the combinations of the primers of the present invention can accurately detect the *ctx* gene irrespective of the source, serological type and biological type of the strains. Heat extracts of the following strains are used as the template DNA solutions:

Lanes 1 to 3: *Vibrio cholerae* (El Tor - Ogawa type, the *ctx* gene positive strain)
Lanes 4 to 6: *Vibrio cholerae* (El Tor - Inaba type, the *ctx* gene positive strain)
Lane 7: *Vibrio cholerae* (Classical- Ogawa type, the *ctx* gene positive strain)
Lane 8: *Vibrio cholerae* (Classical- Inaba type, the *ctx* gene positive strain)
Lanes 9 to 10: *Vibrio cholerae* (non-O1, the *ctx* gene positive strain)
Lane 11: *Vibrio cholerae* (El Tor - Ogawa type, the *ctx* gene negative strain)
Lane 12: *Vibrio cholerae* (El Tor - Inaba type, the *ctx* gene negative strain)
Lane 13: Enterotoxigenic *Escherichia coil* (Thermolabile enterotoxin gene positive strain)

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to *Vibrio cholerae* having the *ctx* gene, the genes of other clinically important pathogenic bacteria are examined with the method of the present invention. The same procedure as used in Experiment 1 is followed, except for the method of preparation of specimens.

Preparation of specimens

Each strain listed in Table 17 is treated in the same manner as in Experiment 2 of Example 5.

Results

Table 17 shows the results of the test using a combination of primers of the present invention. PCR using the primers does not amplify DNAs of any other pathogenic strains tested. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with the DNAs of *Vibrio cholerae* having the *ctx* gene. Similar results are obtained also for the other combination of primers of the present invention which is not listed in Table 17.

Table 17. Reactivity with DNA of bacteria other than Vibrio cholerae

No	Strains		+/−*
1	Bacillus cereus	ATCC 14579	—
2	B. subtilis	JCM 1465	—
3	Staphylococcus aureus	JCM 2413	—
4	S. epidermidis	JCM 2414	—
5	Salmonella typhimurium	IFO 12529	—
6	S. enteritidis	IFO 3163	—
7	Clostridium perfringens	ATCC 12917	—
8	Vibrio fluvialis	JCM 3752	—
9	Campylobacter jejuni	JCM 2013	—
10	C. coli	JCM 2529	—
11	Escherichia coli	JCM 1649	—
12	Yersinia enterocolitica	ATCC 9610	—
13	Shigella dysenteriae	ATCC 9361	—
14	S. flexneri	ATCC 29903	—
15	S. sonnei	ATCC 29930	—
16	Bacteroides fragilis	ATCC 23745	—
17	B. vulgatus	JCM 5826	—
18	Enterococcus faecalis	JCM 5803	—
19	Klebsiella pneumoniae	JCM 1662	—
20	Proteus vulgaris	JCM 1668	—
21	Citrobacter freundii	ATCC 33128	—
22	Streptococcus pyogenes	ATCC 12344	—
23	S. pneumoniae	ATCC 33400	—
24	Haemophilus influenzae	ATCC 33391	—
25	Proteus mirabilis	ATCC 29906	—
26	Neisseria gonorrhoeae	ATCC 19424	—
27	N. meningitidis	ATCC 13077	—
28	Listeria monocytogenes	ATCC 15313	—
29	Lactobacillus acidophilus	JCM 1132	—
30	Bifidobacterium adolescentis	JCM 1275	—
31	Fusobacterium nucleatum	ATCC 2558	—
32	Propionibacterium acnes	ATCC 6919	—
33	Veillonella atypica	ATCC 17744	—
34	Pseudomonas aeruginosa	IFO 12689	—
35	Corynebacterium diphtheriae	JCM 1310	—
36	Peptostreptococcus anaerobius	ATCC 27337	—
37	Human placental DNA		—

Note) * + : reactive
− : nonreactive

Example 10: Detection of *Clostridium perfringens* having the enterotoxin gene

[Experiment 1]

Preparation of specimens

The strains of *Clostridium perfringens* used are 11 strains isolated from patients, and provided by institutes where each strain is stored. Each strain is inoculated to GAM broth (manufactured by Nissui Pharmaceutical Co., Ltd.) and subjected to overnight shaking culture at 37°C under anaerobic conditions. Each culture broth is diluted 10 folds with 10 mM Tris-HCl buffer, pH 7.5, and heated at 95°C for 10 minutes, followed by centrifugation to use the supernatant as a specimen.

Synthesis of primers

As primers for amplifying the enterotoxin gene of *Clostridium perfringens*, the above-described oligonucleotides SEQ ID NO:27 to SEQ ID NO:35 are selected based upon the known base sequences of the enterotoxin gene of *Clostridium perfringens* [Maruke van Damme-Jongsten, Antonie van Leeuwenhoek, 56, 181-190(1989)], and chemically synthesized by the same method as in Experiment 1 of Example 1.

PCR

PCR is carried out under the same reaction conditions as in Example 1 except that any one of the following oligonucleotide combinations is used:

Primer (1) + primer (2) = Oligonucleotide SEQ ID NO:27 + Oligonucleotide SEQ ID NO:32;
Oligonucleotide SEQ ID NO:28 + Oligonucleotide SEQ ID NO:33;
Oligonucleotide SEQ ID NO:29 + Oligonucleotide SEQ ID NO:33;
Oligonucleotide SEQ ID NO:30 + Oligonucleotide SEQ ID NO:34; and
Oligonucleotide SEQ ID NO:31 + Oligonucleotide SEQ ID NO:35.

Detection

Agarose gel electrophoresis

To detect the amplified nucleotide fragments in the reaction solution, agarose gel electrophoresis is carried out by the same procedure as in Example 1.

Figure 3 shows a part of the electrophoretic results. The upper part of the figure shows the results with Oligonucleotide SEQ ID NO:28 + Oligonucleotide SEQ ID NO:33; and the lower part, the results with Oligonucleotide SEQ ID NO:29 + Oligonucleotide SEQ ID NO:33. In the figure, M indicates the molecular weight marker; and lanes 1 to 13 respectively indicate ATCC 12925(lane 1), ATCC 12924(lane 2), ATCC 12922(lane 3), ATCC 12920(lane 4), ATCC 12916(lane 5), ATCC 12915(lane 6), ATCC 12918(lane 7), ATCC 12919(lane 8), ATCC 12921(lane 9), JCM 1296(lane 10), JCM 1416(lane 11), JCM 1382(lane 12), and TE (negative control, lane 13).

Southern blot hybridization test

A southern blot hybridization test is carried out using an oligonucleotide probe specific to the enterotoxin gene of *Clostridium perfringens* according to the method described by Tada et al. [Tada, J. et al. Mol. Cell. Probe., 6, 477 (1992)].

Reversed passive latex agglutination (RPLA) test

A commercially available RPLA kit for detection of *Clostridium perfringens* enterotoxin (PET-RPLA "SEIKEN" produced by DENKA SEIKEN) is purchased. Specimens are prepared and tested according to the instruction manual attached.

Results

The base sequences of the enterotoxin gene of *Clostridium perfringens* have already been determined. Therefore, the length of the nucleotide fragments amplified by PCR using the oligonucleotides of the present invention as primers can easily be estimated. Specifically, when the oligonucleotides SEQ ID NO:27 and SEQ ID NO:32 of the present invention are used in combination, a nucleotide fragment of 473 bases (or a nucleotide duplex of 473 base pairs) should be amplified. When the estimated length accords with the length of the amplified nucleotide fragment, it is judged that PCR using the combination of the primers accurately amplifies the target region in the enterotoxin gene, and that the bacterial strain in the specimen has the enterotoxin gene. The results obtained from the agarose gel electrophoresis and from the RPLA test with the 11 test strains are given in Table 18.

Table 18.

	Strains	Results of RPLA	Combination of primers and length of amplified DNA (No. of b.p.)*				
			27+32** 473	28+33** 456	29+33** 421	30+34** 267	31+35** 156
01	Clostridium perfringens ATCC 12915	+***	+	+	+	+	+
02	Clostridium perfringens ATCC 12916	+	+	+	+	+	+
03	Clostridium perfringens ATCC 12917	+	+	+	+	+	+
04	Clostridium perfringens ATCC 12918	+	+	+	+	+	+
05	Clostridium perfringens ATCC 12919	−	−	−	−	−	−
06	Clostridium perfringens ATCC 12920	+	+	+	+	+	+
07	Clostridium perfringens ATCC 12921	−	−	−	−	−	−
08	Clostridium perfringens ATCC 12922	+	+	+	+	+	+
09	Clostridium perfringens ATCC 12924	+	+	+	+	+	+
10	Clostridium perfringens ATCC 12925	+	+	+	+	+	+
11	Clostridium perfringens JCM 3816	−	−	−	−	−	−

Note) * + : DNA of estimated length is amplified.
− : DNA of any length is not amplified.

** Numerals refer to SEQ ID NOs.

*** + : Agglutination: Enterotoxin is produced.
− : No agglutination: Enterotoxin is not produced.

In the Southern blot hybridization test, it is confirmed that the nucleotide fragments amplified with a combination of the primers of the present invention is a part of the enterotoxin gene sequences. The results are shown in Figure 4. Figure 4 corresponds to Figure 3. In the figure, M indicates the molecular weight marker; and lanes 1 to 13 respectively indicate ATCC 12925(lane 1), ATCC 12924(lane 2), ATCC 12922-(lane 3), ATCC 12920(lane 4), ATCC 12916(lane 5), ATCC 12915(lane 6), ATCC 12918(lane 7), ATCC 12919(lane 8), ATCC 12921(lane 9), JCM 1296(lane 10), JCM 1416(lane 11), JCM 1382(lane 12), and TE (negative control, lane 13).

These results indicate that PCR using the primers of the present invention is capable of accurately amplifying the enterotoxin gene in PCR and that *Clostridium perfringens* having the enterotoxin gene can be detected with high accuracy by using the oligonucleotides of the present invention.

[Experiment 2]

To determine whether the results obtained in Experiment 1 are specific to *Clostridium perfringens* having the enterotoxin gene, the reactivity of the primers of the present invention with the DNAs of other Clostridium species and other clinically important pathogenic bacteria is examined. The same procedure as used in Experiment 1 is followed, except for the method of preparation of specimens.

Preparation of specimens

Each strain listed in Tables 19 and 20 is treated in the same manner as in Experiment 2 of Example 5.

Results

Tables 19 and 20 show the results of the test using some of the combinations of primers of the present invention. All the combinations of the primers listed in the tables do not show any amplification of DNAs of other strains including pathogenic strains in PCR. It can therefore be concluded that the oligonucleotide primers of the present invention selectively react with the enterotoxin gene of *Clostridium perfringens*.

Table 19.

	Strains	Combination of primers and length of amplified DNA (No. of b.p.)*				
		27+32** 473	28+33** 456	29+33** 421	30+34** 267	31+35** 156
01	Clostridium absonum ATCC 27555	−	−	−	−	−
02	Clostridium barati JCM 1382	−	−	−	−	−
03	Clostridium bifermentans ATCC 638	−	−	−	−	−
04	Clostridium butyricum JCM 1391	−	−	−	−	−
05	Clostridium difficile JCM 1296	−	−	−	−	−
06	Clostridium fallax JCM 1398	−	−	−	−	−
07	Clostridium histolyticum JCM 1403	−	−	−	−	−
08	Clostridium novyi JCM 1406	−	−	−	−	−
09	Clostridium sordellii JCM 3814	−	−	−	−	−
10	Clostridium sphenoides JCM 1415	−	−	−	−	−
11	Clostridium spiroforme JCM 1432	−	−	−	−	−
12	Clostridium sporogenes JCM 1416	−	−	−	−	−
13	Clostridium tertiium JCM 6289	−	−	−	−	−

Note) * + : DNA of estimated length is amplified.
− : DNA of any length is not amplified.

** Numerals refer to SEQ ID NOs.

Table 20.

	Strains		Combination of primers and length of amplified DNA (No. of b.p.)*				
			27+32** 473	28+33** 456	29+33** 421	30+34** 267	31+35** 156
01	Vibrio cholerae	ATCC 25872	−	−	−	−	−
02	Vibrio cholerae type Ogawa	ATCC 9458	−	−	−	−	−
03	Vibrio cholerae type Inaba	ATCC 9459	−	−	−	−	−
04	Vibrio fluvialis	JCM 3752	−	−	−	−	−
05	Vibrio metschnikovii	ATCC 7708	−	−	−	−	−
06	Vibrio mimicus	ATCC 33653	−	−	−	−	−
07	Bacillus cereus	ATCC 14579	−	−	−	−	−
08	Bacillus subtilis	JCM 1465	−	−	−	−	−
09	Staphylococcus aureus	JCM 2413	−	−	−	−	−
10	Staphylococcus epidermidis	JCM 2414	−	−	−	−	−
11	Salmonella typhimurium	IFO 12529	−	−	−	−	−
12	Salmonella enteritidis	IFO 3163	−	−	−	−	−
13	Campylobacter jejuni	JCM 2013	−	−	−	−	−
14	Campylobacter coli	JCM 2529	−	−	−	−	−
15	Escherichia coli	JCM 1649	−	−	−	−	−
16	Yersinia enterocolitica	ATCC 9610	−	−	−	−	−
17	Shigella dysenteriae	ATCC 9361	−	−	−	−	−
18	Shigella flexneri	ATCC 29903	−	−	−	−	−
19	Shigella sonnei	ATCC 29930	−	−	−	−	−
20	Bacteroides fragilis	ATCC 23745	−	−	−	−	−
21	Bacteroides vulgatus	JCM 5826	−	−	−	−	−
22	Enterococcus faecalis	JCM 5803	−	−	−	−	−
23	Klebsiella pneumoniae	JCM 1662	−	−	−	−	−
24	Proteus mirabilis	ATCC 29906	−	−	−	−	−
25	Proteus vulgaris	JCM 1668	−	−	−	−	−
26	Citrobacter freundii	ATCC 33128	−	−	−	−	−
27	Streptococcus pyogenes	ATCC 12344	−	−	−	−	−
28	Streptococcus pneumoniae	ATCC 33400	−	−	−	−	−
29	Haemophilus influenzae	ATCC 33391	−	−	−	−	−
30	Neisseria gonorrhoeae	ATCC 19424	−	−	−	−	−
31	Neisseria meningitidis	ATCC 13077	−	−	−	−	−
32	Listeria monocytogenes	ATCC 15313	−	−	−	−	−
33	Lactobacillus acidophilus	JCM 1132	−	−	−	−	−
34	Bifidobacterium adolescentis	JCM 1275	−	−	−	−	−
35	Fusobacterium nucleatum	ATCC 25586	−	−	−	−	−
36	Propionibacterium acnes	ATCC 6919	−	−	−	−	−
37	Veillonella atypica	ATCC 17744	−	−	−	−	−
38	Pseudomonas aeruginosa	IFO 12689	−	−	−	−	−
39	Corynebacterium diphtheriae	JCM 1310	−	−	−	−	−
40	Peptostreptococcus anaerobius	ATCC 27337	−	−	−	−	−

Note) * + : DNA of estimated length is amplified.
− : DNA of any length is not amplified.

** Numerals refer to SEQ ID NOs.

The agarose gel electrophoresis used in the above examples of the present invention can differentiate nucleotide fragments from one another which are different in length by 5-10 bases (base pairs) for nucleotide fragments of not more than 100 bases (base pairs), and by 10-20 bases (base pairs) for nucleotide fragments of 100-500 bases (base pairs). In addition, the use of other gel material such as acrylamide makes it possible to improve the precision in measuring the length of nucleotide fragment. Thus, the reliability of the selective detection of the target gene in the present invention can further be increased.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii)NUMBER OF SEQUENCES: 35

(2) INFORMATION FOR SEQ ID NO:1:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 19 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Shigella dysenteriae* type 1

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAACACTGGA TGATCTCAG 19

(2) INFORMATION FOR SEQ ID NO:2:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 18 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Shigella dysenteriae* type 1

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCCCTCAAC TGCTAATA 18

(2) INFORMATION FOR SEQ ID NO:3:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Shigella dysenteriae, Shigella flexneri, Shigella boydii, and Shigella sonnei*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGTATCACAG ATATGGCATG C 21

(2) INFORMATION FOR SEQ ID NO:4:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Shigella dysenteriae, Shigella flexneri, Shigella boydii, and Shigella sonnei*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCCGGAGATT GTTCCATGTG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Shigella dysenteriae, Shigella*

flexneri, Shigella boydii, and Shigella sonnei

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAAGATTTAA CCTTCGTCAA CC 22

(2) INFORMATION FOR SEQ ID NO:6:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Shigella dysenteriae, Shigella flexneri, Shigella boydii, and Shigella sonnei*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTTCTCGGA TGCTATGCTC 20

(2) INFORMATION FOR SEQ ID NO:7:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 18 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Salmonella spp.*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCGGAGAGG GCGTCATT 18

(2) INFORMATION FOR SEQ ID NO:8:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Salmonella spp.*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAACGACTC ATTAATTACC G 21

(2) INFORMATION FOR SEQ ID NO:9:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 18 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Salmonella spp.*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATCTGGTCGC CGGGCTGA 18

(2) INFORMATION FOR SEQ ID NO:10:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 18 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Salmonella spp.*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCATCGCGCA CACGGCTA 18

(2) INFORMATION FOR SEQ ID NO:11:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 19 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Salmonella spp.*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCGAGCAGT TTGTCTGTC 19

(2) INFORMATION FOR SEQ ID NO:12:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 19 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Escherichia coli*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAACACTGGA TGATCTCAG 19

(2) INFORMATION FOR SEQ ID NO:13:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 18 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Escherichia coli*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCCCTCAAC TGCTAATA 18

(2) INFORMATION FOR SEQ ID NO:14:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Escherichia coli*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATCAGTCGTC ACTCACTGGT 20

(2) INFORMATION FOR SEQ ID NO:15:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 19 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Escherichia coli*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCAGTTATCT GACATTCTG 19

(2) INFORMATION FOR SEQ ID NO:16:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Escherichia coli*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGTTTACGTT AGACTTTTCG AC 22

(2) INFORMATION FOR SEQ ID NO:17:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 19 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Escherichia coli*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGGACAGTAG TTATACCAC 19

(2) INFORMATION FOR SEQ ID NO:18:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 19 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Escherichia coli*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTGCTGTCAC AGTGACAAA 19

(2) INFORMATION FOR SEQ ID NO:19:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Staphylococcus aureus*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCTTTAAAAG TTAAGGTTCA TG 22

(2) INFORMATION FOR SEQ ID NO:20:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Staphylococcus aureus*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCCAAAGTT CGATAAAAAA C 21

(2) INFORMATION FOR SEQ ID NO:21:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 23 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Staphylococcus aureus*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATTTATAGGT GGTTTTTCAG TAT 23

(2) INFORMATION FOR SEQ ID NO:22:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 23 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Staphylococcus aureus*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTGCTTCTAT AGTTTTTATT TCA 23

(2) INFORMATION FOR SEQ ID NO:23:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Vibrio cholerae*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:23:

TGATGAAATA AAGCAGTCAG GT 22

(2) INFORMATION FOR SEQ ID NO:24:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Vibrio cholerae*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:24:

ACAGAGTGAG TACTTTGACC 20

(2) INFORMATION FOR SEQ ID NO:25:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Vibrio cholerae*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGCACTTCTC AAACTAATTG AG 22

(2) INFORMATION FOR SEQ ID NO:26:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Vibrio cholerae*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATACCATCCA TATATTTGGG AG 22

(2) INFORMATION FOR SEQ ID NO:27:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCTGAGGATT TAAAAACACC 20

(2) INFORMATION FOR SEQ ID NO:28:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:28:

ACCCTCAGTA GGTTCAAGTC 20

(2) INFORMATION FOR SEQ ID NO:29:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATGAAACAGG TACCTTTAGC C 21

(2) INFORMATION FOR SEQ ID NO:30:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGTAATATCT CTGATGATGG AT 22

(2) INFORMATION FOR SEQ ID NO:31:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:31:

TAACTCATAC CCTTGGACTC 20

(2) INFORMATION FOR SEQ ID NO:32:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:32:

GAACCTTGAT CAATATTTCC 20

(2) INFORMATION FOR SEQ ID NO:33:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTAGCAGCAG CTAAATCAAG G 21

(2) INFORMATION FOR SEQ ID NO:34:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:34:

AGTCCAAGGG TATGAGTTAG 20

(2) INFORMATION FOR SEQ ID NO:35:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Clostridium perfringens*

(ix)FEATURE:

(A)IDENTIFICATION METHOD: S

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCATCACCTA AGGACTGTTC 20

Claims

1. A synthetic oligonucleotide of 10 to 30 bases which is complementary to a nucleotide sequence of a gene selected from the group consisting of the *Shiga* toxin gene of *Shigella* species, the *ipa*H gene of *Shigella* species and EIEC, the *inv*E gene of *Shigella* species and EIEC, the *ara*C gene of *Salmonella* species, the Verocytotoxin-1 gene of EHEC or VTEC, the Verocytotoxin-2 gene of EHEC or VTEC, the toxic shock syndrome toxin-1 gene of *Staphlylococcus aureus*, the *ctx* gene of *Vibrio cholerae*, and the enterotoxin gene of *Clostridium perfringens*.

2. A synthetic oligonucleotide comprising a nucleotide sequence complementary to the synthetic oligonucleotide of claim 1.

3. A method for detecting a bacterial strain selected from the group consisting of *Shigella* species, EIEC, *Salmonella* species, EHEC, VTEC, *Staphylococcus aureus*, *Vibrio chlolerae*, and *Clostridium perfringens*, wherein the method comprises

(1) hybridizing one primer to a single-stranded target DNA as a template DNA present in a specimen and carrying out a primer extension reaction to give a primer extension product,
(2) denaturing the resulting DNA duplex to separate the primer extension product from the template DNA, the primer extension product functioning as the other template DNA for the other primer,
(3) repeating a cycle of simultaneous primer extension reaction with the two primers, separation of the primer extension products from the templates, and hybridization of primers to amplify a region of the target DNA, in the steps from (1) to (3) said primers being selected from the group consisting of oligonucleotides of claims 1 and 2, and
(4) detecting the amplified nucleotide sequence to determine whether a suspected bacterial strain is present in the specimen.

4. A kit for detection of a bacterial strain, comprising at least a pair of primers selected from the group consisting of oligonucleotides of claims 1 and 2, a thermostable DNA polymerase, and dNTP solutions.

5. The synthetic oligonucleotide according to claim 1, wherein said gene is the *Shiga* toxin gene of *Shigella dysenteriae* type 1, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:1 or Oligonucleotide SEQ ID NO:2.

6. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 5.

7. The synthetic oligonucleotide according to claim 1, wherein said gene is the *ipa*H gene of *Shigella* species and EIEC, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:3 or Oligonucleotide SEQ ID NO:4.

8. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 7.

9. The synthetic oligonucleotide according to claim 1, wherein said gene is the *inv*E gene of *Shigella* species and EIEC, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:5 or Oligonucleotide SEQ ID NO:6.

10. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 9.

11. The method according to claim 3, wherein said target DNA is a gene of *Shigella* species and EIEC and said primers are selected from the group consisting of oligonucleotides of claims 5 to 10.

12. The kit according to claim 4, wherein said pair of primers are selected from the group consisting of oligonucleotides of claims 5 to 10 for detection of *Shigella* species and EIEC.

13. The synthetic oligonucleotide according to claim 1, wherein said gene is the *ara*C gene of *Salmonella* species, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of the oligonucleotide selected from the group consisting of Oligonucleotide SEQ ID NO:7, Oligonucleotide SEQ ID NO:8, Oligonucleotide SEQ ID NO:9, Oligonucleotide SEQ ID NO:10, and Oligonucleotide SEQ ID NO:11.

14. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 13.

15. The method according to claim 3, wherein said target DNA is a gene of *Salmonella* species and said primers are selected from the group consisting of oligonucleotides of claims 13 and 14.

16. The method according to claim 15, wherein said two primers are selected from the following oligonucleotide combinations: a combination in which one oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:7 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:8; a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:9 and the other comprises at least 10 consecutive bases of

Oligonucleotide SEQ ID NO:10; and a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO: 11 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO: 8.

17. The kit according to claim 4, wherein said pair of primers are selected from the group consisting of oligonucleotides of claims 13 and 14 for detection of *Salmonella* species.

18. The synthetic oligonucleotide according to claim 1, wherein said gene is the Verocytotoxin-1 gene of EHEC or VTEC, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:12 or Oligonucleotide SEQ ID NO:13.

19. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 18.

20. The synthetic oligonucleotide according to claim 1, wherein said gene is the Verocytotoxin-2 (VT2) gene or its variant gene (V2vha, VTvhb or VTvp1) of EHEC or VTEC, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:14 or Oligonucleotide SEQ ID NO:15.

21. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 20.

22. The synthetic oligonucleotide according to claim 1, wherein said gene is the Verocytotoxin-1 gene or the Verocytotoxin-2 (VT2) gene or a variant gene of the VT2 gene (V2vha, VTvhb or VTvp1) of EHEC or VTEC, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of the oligonucleotide selected from the group consisting of Oligonucleotide SEQ ID NO:16, Oligonucleotide SEQ ID NO:17, and Oligonucleotide SEQ ID NO:18.

23. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 22.

24. The method according to claim 3, wherein said target DNA is a gene of EHEC or VTEC and said primers are selected from the group consisting of oligonucleotides of claims 18 to 23.

25. The method according to claim 24, wherein said two primers are selected from the following oligonucleotide combinations: a combination in which one oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:12 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:13; a combination in which one oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:14 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:15; a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:16 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:18; and a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO: 17 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO: 18.

26. The kit according to claim 4, wherein said pair of primers are selected from the group consisting of oligonucleotides of claims 18 to 23 for detection of EHEC or VTEC.

27. The synthetic oligonucleotide according to claim 1, wherein said gene is the toxic shock syndrome toxin-1 gene of *Staphylococcus* aureus, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of the oligonucleotide selected from the group consisting of Oligonucleotide SEQ ID NO:19, Oligonucleotide SEQ ID NO: 20, Oligonucleotide SEQ ID NO:21, and Oligonucleotide SEQ ID NO:22.

28. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 27.

29. The method according to claim 3, wherein said target DNA is a gene of *Staphylococcus aureus* and said primers are selected from the group consisting of oligonucleotides of claims 27 and 28.

30. The method according to claim 29, wherein said two primers are selected from the following oligonucleotide combinations: a combination in which one oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:20 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:21; a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:19 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:22; and a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO: 20 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO: 22.

31. The kit according to claim 4, wherein said pair of primers are selected from the group consisting of oligonucleotides of claims 27 and 28 for detection of *Staphylococcus aureus*.

32. The synthetic oligonucleotide according to claim 1, wherein said gene is the cholera toxin gene of *Vibrio cholerae*, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of the oligonucleotide selected from the group consisting of Oligonucleotide SEQ ID NO:23, Oligonucleotide SEQ ID NO:24, Oligonucleotide SEQ ID NO:25, and Oligonucleotide SEQ ID NO:26.

33. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 32.

34. The method according to claim 3, wherein said target DNA is a gene of *Vibrio cholerae* and said primers are selected from the group consisting of oligonucleotides of claims 32 and 33.

35. The method according to claim 34, wherein said two primers are selected from the following oligonucleotide combinations: a combination in which one oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:23 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:25; and a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:24 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:26.

36. The kit according to claim 4, wherein said pair of primers are selected from the group consisting of oligonucleotides of claims 32 and 33 for detection of *Vibrio cholerae*.

37. The synthetic oligonucleotide according to claim 1, wherein said gene is the enterotoxin gene of *Clostridium perfringens*, and wherein the synthetic oligonucleotide comprises at least 10 consecutive bases of the oligonucleotide selected from the group consisting of Oligonucleotide SEQ ID NO:27, Oligonucleotide SEQ ID NO:28, Oligonucleotide SEQ ID NO:29, Oligonucleotide SEQ ID NO:30, Oligonucleotide SEQ ID NO:31, Oligonucleotide SEQ ID NO:32, Oligonucleotide SEQ ID NO:33, Oligonucleotide SEQ ID NO:34, and Oligonucleotide SEQ ID NO:35.

38. The synthetic oligonucleotide according to claim 2, wherein said nucleotide sequence is complementary to the synthetic oligonucleotide of claim 37.

39. The method according to claim 3, wherein said target DNA is a gene of *Clostridium perfringens* and said primers are selected from the group consisting of oligonucleotides of claims 37 and 38.

40. The method according to claim 39, wherein said two primers are selected from the following oligonucleotide combinations: a combination in which one oligonucleotide comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:27 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:32; a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:28 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:33; a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:29 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:33; a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:30 and the other comprises at least 10 consecutive bases of

Oligonucleotide SEQ ID NO:34; and a combination in which one comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:31 and the other comprises at least 10 consecutive bases of Oligonucleotide SEQ ID NO:35.

41. The kit according to claim 4, wherein said pair of primers are selected from the group consisting of oligonucleotides of claims 37 and 38 for detection of *Clostridium perfringens*.

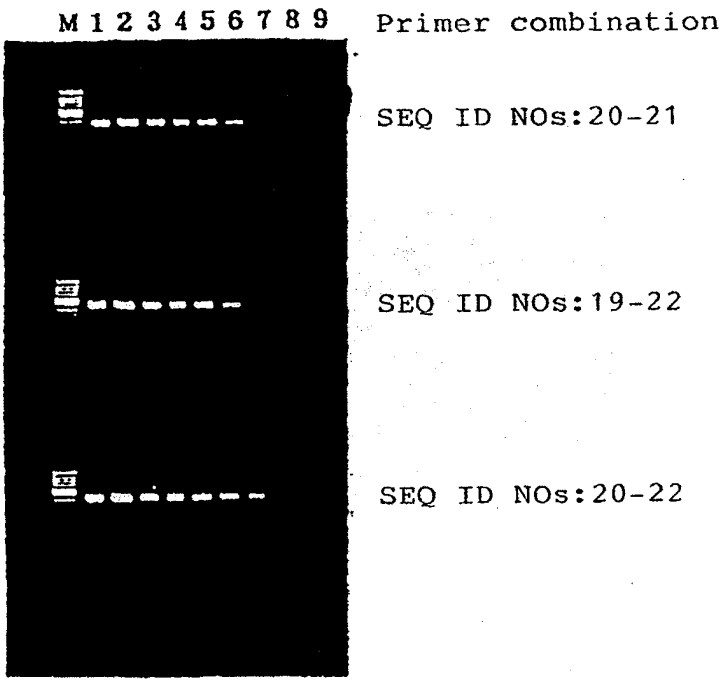
M 1 2 3 4 5 6 7 8 9
Primer combination
SEQ ID NOs:20-21
SEQ ID NOs:19-22
SEQ ID NOs:20-22

FIG. 1

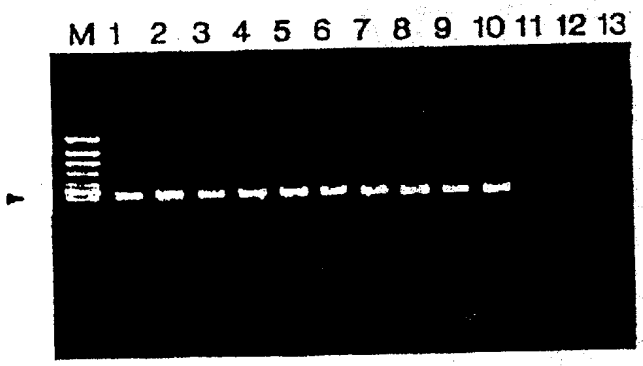

FIG. 2

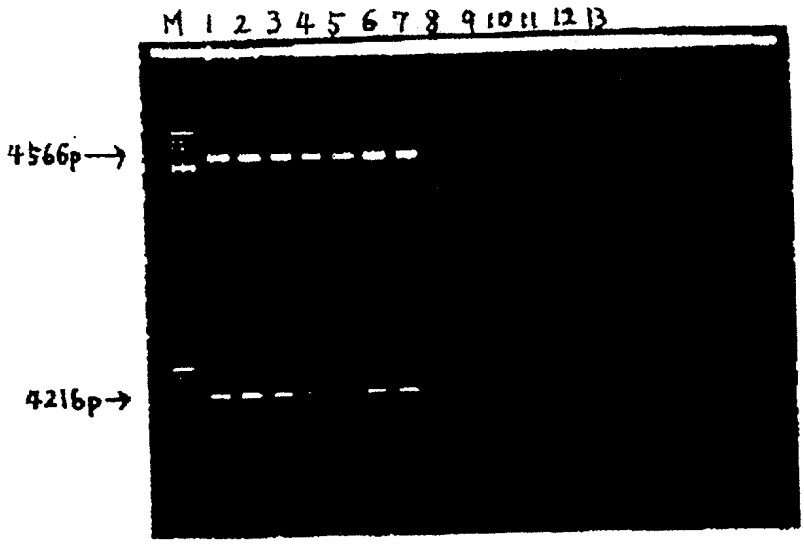

FIG. 3

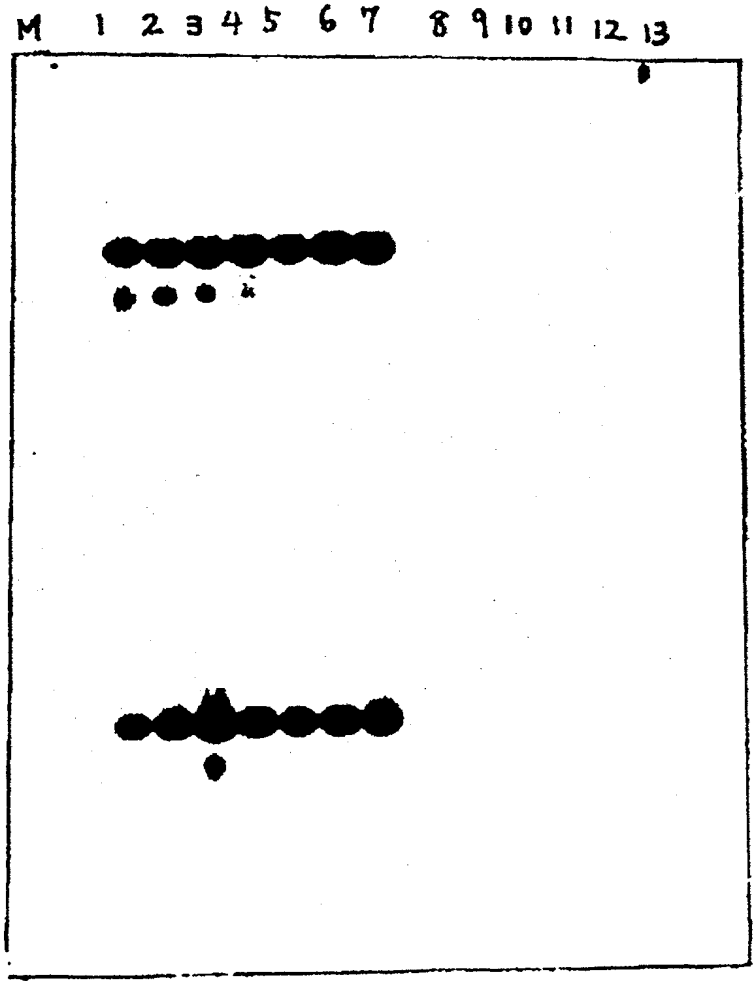

FIG. 4